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SHINFIELD READING ENGLAND

DAIRY SCIENCE ABSTRACTS

COMPILED FROM WORLD LITERATURE

The Bureau is a clearing house of world information on all matters concerned with dairy husbandry, technology and science, covering the milk of all mammals, including human milk, and dealing with the many dairy processes and products. All inquiries, editorial and technical matters should be addressed to the Director at Shinfield, Reading.

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Owing to increased paper and printing charges, it will be necessary to raise the annual subscription in January 1953, Vol. XV, to the rates given below.

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COMMONWEALTH AGRICULTURAL BUREAUX
CENTRAL SALES BRANCH, FARNHAM ROYAL, BUCKS., ENGLAND

THE COSTANTINO GORINI FOUNDATION

A foundation has been created honouring the name of Costantino GORINI, well-known dairy bacteriologist who died in September, 1950 in his native city of Milan, Italy. It was formed under the auspices of the Lombardo Institute of Sciences and Letters of Milan for the purpose of assisting research workers engaged in dairy science and technology, especially in the field of microbiology.

Periodically an international competition will be held and a prize awarded the candidate who has demonstrated the best results in improving the production and uses of milk and dairy products in the interest of human nutrition.

Competition is now open and will close on December 31, 1952. Entries should be sent to INSTITUT LOMBARDO, VIA BRERA 28, MILAN. An entry shall consist of a letter of application, supporting reprints of published work and an account of the research work completed since 1949. The reprints and the letter of application may be written in one of the following languages: Italian, French, English, Spanish or German. The account of the work itself must, however, be written in Italian or French.

The prize is 100,000 liras.

637.128.64

480. THE ROLE OF MICRO-ORGANISMS IN DYE-REDUCTION AND KEEPING-QUALITY TESTS

II. THE EFFECT OF MICRO-ORGANISMS WHEN ADDED TO MILK IN PURE AND MIXED CULTURE

BY ELLEN I. GARVIE AND A. ROWLANDS
National Institute for Research in Dairying, University of Reading

(With 1 Figure)

In Part I the bacteria predominating at the time of dye reduction at 37·5° C. and at the end-point of the clot-on-boiling test at 22° C. were discussed. During incubation of milk at 22 and 37·5° C. enrichment of some types of bacteria was shown to occur. To obtain further information about the part played by some of these bacteria in dye reduction and milk spoilage, their action, when added to sterile aseptically drawn milk in pure and mixed culture, has been studied.

Earlier investigations by Wilson (1), Jensen (2), Hobbs (3) and Thomé (4) have shown that bacteria vary in their ability to reduce methylene blue at 37·5° C. Generally speaking, some micrococci, staphylococci, the *coli-aerogenes* bacteria and some streptococci were found to be active, while other organisms, notably some streptococci and the achromobacteria were relatively inert. Wilson (1) ascribed the failure of some cultures to reduce the dye at 37·5° C. to their inability to grow at this temperature. According to Egdell & Bird (5) *Streptococcus lactis*, *Bacillus cereus*, and the *coli-aerogenes* group of bacteria are active in the spoilage of aseptically drawn pasteurized milk at 20° C.; other cultures including some achromobacteria are relatively inert.

Anderson, Meanwell & Wright (6) reported that acid production by streptococci in milk is stimulated in the presence of some aerobic spore-forming bacilli. Frazier & Whittier (7) and Iya & Frazier (8) have reported effects on Eh drift and gas formation when *Str. lactis* is grown with strains of *coli-aerogenes* bacteria. Thomé (4) concluded that the effect on the reduction of methylene blue at 37·5° C. of mixed cultures of streptococci, micrococci and the *coli-aerogenes* groups was essentially the same as when they were present in milk in pure culture.

EXPERIMENTAL METHODS

Source and collection of the milk samples. Milk from four healthy heifers was used. The animals were selected from a herd regularly examined for mastitis by Mr F. K. Neave of the National Institute for Research in Dairying, and only animals which had been consistently free from udder infection were chosen. Because of the volume of milk required (2000 ml. at each sampling) it was considered impracticable to withdraw it aseptically by hand and, therefore, the cows were milked by a bucket-type machine reserved for this work. Before use the machine was washed in the laboratory and autoclaved (10 lb. for 10 min.). It was then assembled in the laboratory. Approximately

500 ml. of the bulk evening milk from each of the four cows was mixed, and within ½ hr. of sampling the milk was distributed in 50 ml. quantities into sterile 8 × 1 in. test tubes, pasteurized in a water-bath at 61 ± 0·5° C. for 20 min. and immediately cooled in iced water. The tubes were maintained at below 5° C. until required.

Observations on pure cultures

Inoculation of the milk. The method of preparation of the milk is represented diagrammatically in Fig. 1. A 12 hr. culture (in Lemco broth containing 1% lactose) of the test

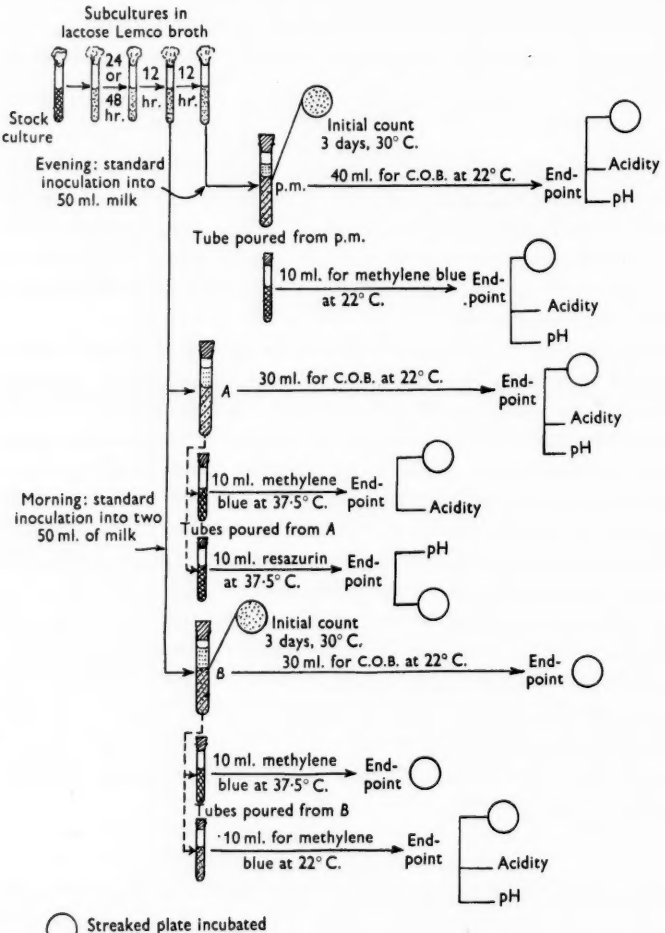

Fig. 1. The method of pure culture experiment.

organisms was used to inoculate two 50 ml. quantities of milk (*A* and *B*) at approximately 9 a.m. and one 50 ml. quantity at approximately 9 p.m. The broth culture used to inoculate the milk was either a third or a fourth transfer through broth and was always itself prepared from a 12 hr. culture. The inoculum was calculated to give approximately

2,000,000 living organisms/ml. of the milk; the size of the inoculum was verified by preparing plate counts of the milk on yeastrel milk agar (Ministry of Health (9)) incubated for 3 days at 30° C. From each of the tubes *A* and *B* two 10 ml. quantities were removed, those from tube *A* were used for the resazurin and methylene-blue reduction tests at 37·5° C., and those from tube *B* for the methylene-blue reduction test at 37·5 and 22° C. The remainder of the milk was kept at 22° C. for determination of keeping quality using precipitation with 68% alcohol and clot-on-boiling to determine end-point. Thus the methylene-blue reduction at 37·5° C. and the keeping quality at 22° C. were carried out in duplicate.

In the evening only one 10 ml. quantity was removed from the 'p.m.' tube. This was used for the methylene-blue reduction test at 22° C.; the remainder of the milk was used for the keeping quality test at 22° C. By putting up samples for the methylene blue and keeping quality tests at 22° C. at intervals of 12 hr. it was hoped to obtain continuous reading over a period of 72 hr. without having recourse to night observations.

Acidity and pH determinations were made at the time of the end-point of the tests for a number of the cultures studied.

The dye reduction and keeping quality tests were made as described by Garvie & Rowlands (10).

The purity of the cultures at test end-point was checked by preparing streak plates on yeastrel milk agar.

Observations on mixed cultures

Inoculation of the milk. Milk obtained and prepared as already described was inoculated from two 12 hr. pure broth cultures to give the desired numbers of the two cultures/ml. of the milk. The size of the inoculum was verified by preparing plate counts from control milk tubes inoculated with only one of the cultures.

Estimation of dye reduction and keeping quality. The procedure was exactly as described for the pure cultures.

The numbers of each type present at end-point were determined using the Miles & Misra (11) technique on yeast dextrose agar. In general, the appearance of surface colonies permitted a reliable count to be made. Additional counts, using the same technique, were made for staphylococci on mannitol salt agar (Garvie & Rowlands (10)); for the *coli-aerogenes* group on violet red bile agar (Bartram & Black (12)), and for the achromobacteria on nutrient agar.

RESULTS AND DISCUSSION

Activity of pure cultures

A total of 100 cultures was examined and the results are summarized in Table 1.

The reduction of methylene blue and resazurin at 37·5° C. In general, the times to reduction of methylene blue and resazurin were in good agreement. There were two notable exceptions. The first was a group B *Streptococcus* which failed to reduce resazurin in 9 hr. but which reduced methylene blue in 6 and 3½ hr. in replicate tests. These results were not further investigated. This culture was the only group B *Streptococcus* tested which reduced methylene blue within the period of observations. The second culture was also a *Streptococcus*, 32. R. 2; it reduced resazurin more rapidly than methylene blue. It was found that *Streptococcus* 32. R. 2 was unable to grow as rapidly in the presence of methylene blue as in the presence of resazurin (see Appendix). This organism

could not be classified serologically, but on the results of physiological tests appeared to be related to group D. It was the only culture encountered whose proliferation was somewhat retarded in the presence of methylene blue at the concentration used in the reduction test.

The time to reduction of the two dyes at 37·5° C. was observed to be relatively long with the streptococci of groups B, C and E, the heterofermentative streptococci and some strains of group N. These last-mentioned cultures were not *Str. lactis* strains, and like the heterofermentative types did not appear to grow well at 37·5° C.

The group D strains examined and the *Str. lactis* strains from group N, the *coli-aerogenes* bacteria, staphylococci and some micrococci were active in dye reduction, while the achromobacteria, chromobacteria, microbacteria and other micrococci were relatively inert. A temperature of 37·5° C. is probably too high for the active growth of all these last-mentioned organisms. It would seem then that inability to reduce the dye at 37·5° C. is at least in part associated with inability to grow at this temperature.

The cultures which were inactive towards the dyes at 37·5° C. were for the most part isolated from the milk samples initially, or at the end-point of the clot-on-boiling test. In the former case it would appear that although these cultures formed a considerable proportion of the milk initially, they failed to multiply rapidly and were overgrown by the time of reduction of the dyes and in fact played little part in reduction. In the latter case the cultures failed to proliferate at 37·5° C. and presumably did not contribute materially to dye reduction.

The reduction of methylene blue at 22° C. With the exception of the two group C cultures all the streptococci examined reduced methylene blue fairly rapidly at 22° C. The *coli-aerogenes* strains examined, the staphylococci and most of the micrococci, the Gram-positive rods, the achromobacteria and chromobacteria all reduced dyes actively at 22° C.; the microbacteria were less active.

It was rather surprising to find that the group B and E cultures which grow well at 37·5° C. and reduce methylene blue slowly at this temperature are quite active at 22° C. Even more surprising, perhaps, is the fact that more group B streptococci were isolated from milk initially from plates incubated at 22° C. than at 37·5° C. (Garvie & Rowlands (10)). Therefore, it is possible that in raw milk the group B streptococci may play some part in dye reduction at 22° C. Jones & Davis (13) have also noted the inability of *Str. agalactiae* to reduce dyes at 37·5° C. although having a marked effect on the keeping quality as measured by the clot-on-boiling test at a lower temperature. Strains of group N streptococci, micrococci and achromobacteria behave similarly, and it seems that discrepancies between dye results at 37·5 and 22° C. may, to some extent, be associated with the incidence of these types in different milk.

In the earlier work described (Garvie & Rowlands (10)), the flora at the end-point of the methylene-blue reduction test at 22° C. was not examined and it could not therefore be established whether any of these cultures constituted an appreciable portion of the flora at that time. Wilson (1) found streptococci at the end-point of the methylene-blue reduction test at 17·5° C. but not as frequently as the achromobacteria or *Pseudomonas fluorescens*.

Effect on keeping quality at 22° C. Generally speaking, the end-point at 22° C. using the 68% alcohol test was followed shortly by the end-point of the clot-on-boiling test. The notable exception was *Str. faecalis* 775, where a long lag occurred between the end-points. While a positive result was obtained with the alcohol test in 27 hr. the end-point of the clot-on-boiling test was not reached until 60 hr. Egdell & Bird (5) report the same

Table 1. *The effect of pure cultures of bacteria on dye-reduction tests at 37·5 and 22° C. and on keeping quality at 22° C.*

Description of culture	Type of culture	Name of culture	No. tested	Dye-reduction time: 37·5° C. Resazurin: <3½ hr.	3½–4½ hr.	5–6 hr.	6½–9 hr.	>9 hr.	37·5° C. Methylene blue: <3½ hr.	3½–4½ hr.	5–6 hr.	6½–9 hr.	>9 hr.	22° C. Methylene blue: <12 hr.	12–24 hr.	27–36 hr.	39–48 hr.	51–60 hr.	63–70 hr.	>70 hr.	Keeping quality 22° C. 68% alcohol: <12 hr.	12–24 hr.	27–36 hr.	39–48 hr.	51–60 hr.	63–70 hr.	>70 hr.	C.O.B.: <12 hr.	12–24 hr.	27–36 hr.	39–48 hr.	51–60 hr.	63–70 hr.	>70 hr.
Streptococci	Group B	—	10	—	—	—	2	7	—	1	1	1	6	—	10	3	—	—	—	—	—	9	5	—	—	—	—	—	7	7	2	—	—	—
	C	—	2	—	—	1	1	1	—	—	1	1	1	—	—	1	2	—	—	—	—	—	1	2	1	—	—	—	—	—	2	2	1	—
	E	—	4	—	—	—	—	4	—	—	—	—	4	—	3	2	1	—	—	—	—	—	3	1	—	1	—	1	—	3	2	—	1	1	—
	D	*Str. faecalis*	2	1	—	—	—	—	2	—	—	—	—	1	2	—	—	—	—	—	—	1	1	—	—	—	—	—	1	—	—	—	1	—	—
		Str. faecalis var. *liquefaciens*	1	1	—	—	—	—	1	—	—	—	—	—	1	—	—	—	—	—	—	1	—	—	—	—	—	—	1	—	—	—	—	—	—
		Str. bovis	1	1	—	—	1	—	1	—	—	1	—	—	1	—	—	—	—	—	—	1	—	—	—	—	—	—	1	1	—	—	—	—	—
		Unclassified	3	1	2	1	2	1	—	2	2	1	2	—	3	1	1	—	—	—	—	2	3	1	—	—	—	—	1	2	2	1	—	—
	Heterofermentative	—	4	—	—	1	—	3	—	—	1	—	3	—	4	—	—	—	—	—	—	4	—	—	—	—	—	—	—	3	1	1	—	1
	Group N	*Str. lactis* vars.	6	1	6	2	—	—	2	5	2	—	—	3	6	—	—	—	—	—	—	6	1	—	—	—	—	—	—	5	2	1	—	—	—
		Other varieties	8	—	2	—	—	8	—	—	2	—	8	7	4	2	—	—	—	—	—	4	7	3	—	—	—	—	2	6	3	1	—	—	—
Coli-aerogenes	*Bact. coli* (type 1)	—	2	2	—	—	—	—	2	—	—	—	—	1	2	—	—	—	—	—	—	2	—	—	—	—	—	—	1	1	—	—	—	—	—
	Intermediate I	—	2	—	2	1	—	—	2	1	—	—	—	2	1	—	—	—	—	—	—	1	2	—	—	—	—	—	2	—	—	—	—	—	—
	Irregular V	—	1	—	1	1	—	—	—	1	—	—	—	—	1	—	—	—	—	—	—	1	—	—	—	—	—	—	1	—	—	—	—	—	—
Achromobacteria	—	—	10	—	—	—	—	10	—	—	—	1	10	3	6	2	1	—	—	1	—	1	—	1	2	3	9	—	1	1	—	—	—	9
Gram-positive rods	—	—	4	—	—	—	1	3	—	—	1	2	3	—	1	2	1	—	1	2	—	—	1	—	1	1	3	—	—	—	1	1	—	4
Chromobacteria	—	—	1	—	—	—	—	1	—	—	—	—	1	—	1	—	—	—	—	—	—	1	—	—	—	—	—	—	—	—	—	—	—	—	1
Microbacteria	—	—	4	—	—	—	—	4	—	—	—	—	4	—	2	2	—	—	—	—	—	—	2	3	2	1	—	—	—	—	2	—	3	1
Micrococci	Staphylococci	—	7	7	—	—	—	—	7	—	—	—	—	2	7	—	—	—	—	—	—	4	4	2	2	1	—	—	1	7	—	1	2	1
	Orange micrococci	—	13	8	6	2	1	—	8	5	4	1	—	5	9	—	2	—	—	—	—	2	3	4	—	2	4	—	—	3	2	5	—	5
	Other micrococci	—	15	5	4	1	2	7	5	3	3	3	6	3	11	8	1	—	—	1	—	4	5	4	1	4	4	—	2	1	5	5	3	5

In compiling Table 1, every result obtained from each culture has been taken into account. Duplicate tests did not always give results falling into the same column in the table, and with some cultures (with the samples at 22° C.) the same is true of the 'A' and 'p.m.' samples. Because of these variations in the test results there is a difference between the number in column 4 and the totals obtained from the succeeding columns.

phenomena when working with the same strain of organism also in aseptically drawn pasteurized milk. They found that after an initial period of rapid growth multiplication virtually ceased, and explain their results on this basis.

The cultures most active in their effect on keeping quality at 22° C. were found amongst the *coli-aerogenes* bacteria, the group N streptococci (including those virtually inert in dye reduction at 37·5° C.) and the group D streptococci. Other streptococci, the staphylococci and other micrococci were less active. The microbacteria and group C strains were very slow in producing an end-point, and the achromobacteria, chromobacteria, Gram-positive rods and some micrococci failed to produce an end-point within the 72 hr. period of observation.

While those cultures which are active in milk spoilage at 22° C. are also active in dye reduction, at this temperature many cultures having very little effect on milk spoilage are active in dye reduction. From the observations of Wilson (1) for the cultures predominating at the time of reduction of methylene blue at 17·5° C. and from our own results (Garvie & Rowlands (10)) with the dominant flora at the end-point of the clot-on-boiling test at 22° C., it seems safe to assume that in milk samples the same organisms are not necessarily the cause of both end-points. These conclusions are amply supported by the results of the pure culture work. While with some cultures, e.g. the streptococci, and the *coli-aerogenes* group, the reduction of methylene blue, the alcohol and the clot-on-boiling test end-point follow each other in fairly rapid succession; with other cultures, e.g. the achromobacteria and some micrococci, the milk fails to precipitate with alcohol or to clot when boiled although methylene blue was rapidly reduced.

It would appear, that these cultures, at 22° C. produce metabolic changes which are apparently distinct from those which are concerned with the spoilage of milk. No studies have been made with the constituents of milk metabolized by the types that are active dye reducers but proved to be inert with regard to milk spoilage as usually measured. Neither the achromobacteria nor chromobacteria ferment lactose and yet these cultures were able to reduce methylene blue in a short time, evidently the breakdown of some constituent of milk other than lactose resulted in the reduction of the dye. With some strains of *Staphylococcus aureus*, milk inoculated from a third-generation broth culture, while reducing methylene blue in a short time, gave an end-point with the clot-on-boiling test in approximately 60 hr.; while milk inoculated from a fourth-generation broth culture gave the same dye reduction time but an end-point to the methylene-blue reduction test in under 36 hr. Evidently with these strains, which are lactose fermenters, the reduction of the dye was not due to the fermentation of the carbohydrate or a rapid end-point to the clot-on-boiling test would have followed in every sample.

The effect of mixed cultures

Based on the observations of the dominant bacterial flora present at the time of the end-point of the clot-on-boiling test in raw milk (Garvie & Rowlands (10)) group N streptococci were apparently the most important group. Accordingly, attention was first given to the effect on dye reduction and keeping quality tests of adding other cultures to *Str. lactis*. Two strains of *Str. lactis* were used.

The other cultures were selected from the *coli-aerogenes* group, the staphylococci and coagulase negative orange micrococci and the achromobacteria. The results are given in Table 2.

Table 2. *Dye reduction and keeping quality tests of mixed cultures*

No. of culture	Strain of organism	Pure culture: Amount of culture inoculated	Pure culture: Reduction time at 37·5° C. Resazurin	Pure culture: Reduction time at 37·5° C. Methylene blue	Pure culture: Reduction time at 22° C. Methylene blue	Pure culture: Time to precipitation with 68% alcohol	Pure culture: Time to C.O.B. (hr. at 22° C.)	Mixed cultures: Amount of *Streptococcus* inoculated	Mixed cultures: Reduction time at 37·5° C. Resazurin	Mixed cultures: Reduction time at 37·5° C. Methylene blue	Mixed cultures: Reduction time at 22° C. Methylene blue	Mixed cultures: Precipitation with 68% alcohol	Mixed cultures: Time to C.O.B. (hr. at 22° C.)	Proportion of *Streptococcus* culture at end-point of: Dye reduction at 37·5° C.	Proportion of *Streptococcus* culture at end-point of: Dye reduction at 22° C.	Clot-on-boiling at 22° C.
27. C. 3	*Bact. coli* (type I)	1×10^6	3	2½	12	21	27	1×10^2	3	2	12	18	24	All coliform	All coliform	1 : 0·45
		1×10^4	5	4½	18	24	33	1×10^4	4	3½	15	15	18	1 : 10	1 : 0·04	1 : 0·03
		—	—	—	—	—	—	1×10^2	4½	4	18	24	27	All coliform	1 : 4	1 : 0·2
		1×10^2	7	6½	24	30	39	1×10^4	5½	5	15	15	18	1 : 1	1 : 0·001	1 : 0·002
		—	—	—	—	—	—	1×10^2	7	6	21	24	27	All coliform	1 : 0·1	1 : 0·045
30. C. 15	*Bact. coli* (Inter. type I)	1×10^6	4	3½	10½	12	21	1×10^2	4	3½	10½	12	21	All coliform	All coliform	1 : 26
		1×10^4	7½	6½	18	21	24	1×10^4	6	6	15	15	18	1 : 0·2	1 : 0·25	1 : 0·2
		—	—	—	—	—	—	1×10^2	7½	6½	18	21	24	1 : 13	1 : 20	1 : 5
		1×10^2	10½	10	21	24	33	1×10^4	8	8	16½	16½	19½	1 : 0·02	1 : 0·05	1 : 0·018
		—	—	—	—	—	—	1×10^2	10	9½	21	24	27	1 : 1·5	1 : 2	1 : 0·4
26. 22. 5	Orange *Micrococcus*	1×10^6	2	2	9	30	48	1×10^2	2	2	12	24	27	All *Micrococcus*	All *Micrococcus*	1 : 0·03
		1×10^4	5	5	30	45	+72	1×10^4	4½	4½	—	—	—	All *Micrococcus*	—	—
		—	—	—	—	—	—	1×10^2	5	5	21	24	27	All *Micrococcus*	1 : 0·08	1 : 0·1
		1×10^2	7½	7½	36	+72	+72	1×10^4	7	7	—	—	—	1 : 0·1	—	—
		—	—	—	—	—	—	1×10^2	7½	7½	24	24	27	All *Micrococcus*	1 : 0·00025	1 : 0·0001
28. D. 6	*Staph. aureus*	1×10^6	2½	2½	12	33	54	1×10^2	2½	2½	12	24	27	All *Streptococcus*	All *Streptococcus*	1 : 0·02
		1×10^4	5½	5½	33	+72	+72	1×10^2	5½	5½	21	24	27	All *Streptococcus*	1 : 0·015	1 : 0·001
		1×10^2	8	8	36	+72	+72	1×10^2	8	8	21	24	27	All *Streptococcus*	1 : 0·0004	1 : 0·0002
27. C. 14	Achromobacteria	1×10^6	+12	+12	18	+72	+72	1×10^2	9½	9½	15	21	24	No achromobacteria recorded on any plates		
		1×10^4	+12	+12	21	+72	+72	1×10^2	+12	+12	18	24	27	Lowest dilution plated 1/100		
		1×10^2	+12	+12	24	+72	+72	1×10^2	+12	+12	18	24	27			
30. C. 14	Achromobacteria	1×10^6	+12	+12	48–60	+72	+72	1×10^2	+11	+11	24	24	27	No achromobacteria recorded on any plates		
		1×10^4	+12	+12	48–60	+72	+72	1×10^2	+11	+11	24	24	27	Lowest dilution plated 1/100		
		1×10^2	+12	+12	+72	+72	+72	1×10^2	+11	+11	24	24	27			
35. C. 9	*Str. lactis*	1×10^6	3	3	9	12	12	—	—	—	—	—	—		—	
		1×10^4	8	8	18	18	21	—	—	—	—	—	—		—	
		1×10^2	+11	+11	15	24	27	—	—	—	—	—	—		—	
27. C. 3	Achromobacteria*	1×10^6	7½	5½	18	+72	+72	1×10^2	5	3½	15	21	24	All achromobacteria	1 : 16	1 : 0·00085
		1×10^4	+11	+11	27	+72	+72	1×10^2	6½	5½	18	24	27	All achromobacteria	1 : 0·37	1 : 0·04
		1×10^2	+11	+11	30	+72	+72	1×10^2	9½	9	21	24	30	1 : 0·00023	1 : 0·41	1 : 0·027
26. D. 12.	*Micrococcus**	1×10^6	1	1	12	+72	+72	1×10^2	1	1	12	24	24	All *Micrococcus*	All *Micrococcus*	1 : 0·1
		1×10^4	+11	+11	42	+72	+72	1×10^2	10	10½	21	24	30	1 : 0·015	1 : 0·17	1 : 0·0023
		1×10^2	+11	+11	+72	+72	+72	1×10^2	10	10½	21	24	30	1 : 0·00046	All *Streptococcus*	
30. D. 2	*Str. lactis*	1×10^2	10	10	21	24	30	—	—	—	—	—	—		—	

* Cultures iced from time of inoculation until 5.00 p.m. then aged overnight at 14° C. Dyes added the following morning and samples held at 37·5° C. until time of reduction. Grown in association with 30. D. 2.

The experiments as designed could only be expected to indicate possible trends in raw milk. The main difficulty was the rapidity with which *Str. lactis* reached end-point with the alcohol and clot-on-boiling tests. Even when only 100 cells/ml. of milk were inoculated the end-point of the clot-on-boiling test was reached in 30 hr. or less, and it was difficult to show any decrease due to the associated culture. It has not been possible to ascribe any of the results obtained with the mixtures to the associated growth of the organisms, but it might appear that both the *coli-aerogenes* bacteria and achromobacteria had some effect in hastening milk spoilage when initially present in large numbers.

From the estimations of the bacteria present in the milk initially, at the time of dye reduction at 37·5 and 22° C. and at the end-point of the clot-on-boiling test, it is possible to gain information regarding the rates of growth of the various cultures at 37·5 and 22° C.

At 37·5° C. the *Bact. coli* (type I), the *Staphylococcus* and the orange *Micrococcus* were apparently growing faster than the *Str. lactis* cultures, and were invariably the dominant flora at the time of dye reduction. The *Str. lactis* was not overgrown to the same extent by the *Bact. coli* (intermediate I), while the other cultures examined were completely overgrown by *Str. lactis*.

At 22° C. the *Str. lactis* was growing faster than any other culture examined and was either the dominant flora by the time of reduction of methylene blue or was present in a greater proportion than in the initial milk. The difference between the rate of growth of *Str. lactis* and *Bact. coli* (intermediate I) was less marked than with the other cultures. By the time the end-point of the clot-on-boiling test had been reached, *Str. lactis* was either the dominant organism or in some cases where it was vastly outnumbered in the initial milk, it formed a substantial part of the flora.

These results are in keeping with the observations on the dominant flora of raw milk at the time of methylene-blue reduction at 37·5° C. and at the end-point of the clot-on-boiling test at 22° C. (Garvie & Rowlands (10)). In raw milk the staphylococci and micrococci tended to become dominant at 37·5° C., particularly in clean milk, while *Str. lactis* tended to become dominant at the end-point of the clot-on-boiling test. In the samples of raw milk examined the numbers of the *coli-aerogenes* bacteria present were estimated by plating on violet red bile agar, and these results showed that the numbers of this group of cultures which were present in the milk initially could not be related to the numbers present at dye reduction at 37·5° C. or at the end-point of the clot-on-boiling test at 22° C.; further, the increase in numbers of the *coli-aerogenes* group at 37·5° C. was not related to the increase at 22° C. A partial explanation of the results obtained with the raw milk samples can be offered in the light of the behaviour of the two *Bact. coli* cultures now studied. While the *Bact. coli* (type I) grew faster than the *Str. lactis* culture at 37·5° C. it grew more slowly at 22° C., the *Bact. coli* (intermediate I) was growing both at 37·5 and at 22° C. at a rate similar to that of *Str. lactis*. In other words, the strain of *Bact. coli* (type I) was growing more rapidly than the strain of *Bact. coli* (intermediate I) at 37·5° C. but more slowly at 22° C. It is suggested, therefore, that the results obtained with raw milk samples will be partly dependent on the type of *coli-aerogenes* contamination of the milk. Malcolm (14) studied the rate of growth of various members of the *coli-aerogenes* group and reported that while some members were more active at 37·5° C., others were more active at 22° C.

Using an inoculum of only 100 cells/ml. of the milk, *Str. lactis* failed to reduce methylene blue at 37·5° C. within the period of observation, and neither cultures 27. C. 14 (achromobacteria) nor 26. D. 12 (*Micrococcus*) were active at 37·5° C. Raw milk samples are never examined by the methylene-blue reduction test at 37·5° C. until they have been aged for several hours at atmospheric temperature. In order to simulate field conditions the portions of the samples inoculated with the *Streptococcus*, and 27. C. 14 and 26. D. 12 which were to be examined by the dye-reduction tests at 37·5° C., were iced from the time of inoculation until 5 p.m. and then held at 14° C. until 9 a.m. the following morning when testing was commenced. In both the pure and mixed cultures the dye was reduced quickly, and the plates prepared from the samples containing the mixed cultures showed only achromobacteria or *Micrococcus* and reduction must have been due mainly to these cultures. Neither grow at 37·5° C. but reduction occurred in 5½ hr. with the achromobacteria and in 1 hr. with the *Micrococcus* in pure culture at 37·5° C. The cultures were held for 17 hr. at 14° C., and reduction at 37·5° C. must have been due to activity during the pre-test storage period and not due to activity at 37·5° C. It must be remembered that the count in these milk samples was large and it would be dangerous to assume to what extent these results can be applied to raw milk samples.

Acidity and pH *values*

Determinations of acidity and pH at the time of dye reduction at 37·5° C. and at the end-point of the clot-on-boiling test, were made on 72 pure culture samples. Very little information could be gained from the results. Four cultures, three micrococci (grouped as other micrococci) and one achromobacteria gave readings at the end-point of the clot-on-boiling test indicative of peptonization and not acidity at end-point. All the other cultures gave readings indicative of developing acidity at the end-point of the clot-on-boiling test.

At the time of dye reduction at 37·5° C. the readings of pH and acidity showed very little variation between cultures and only a slight change from the initial readings.

SUMMARY AND CONCLUSIONS

1. A study of the ability of pure cultures to reduce methylene blue and resazurin at 37·5° C., to reduce methylene blue at 22° C. and to cause milk spoilage as measured by precipitation with 68% alcohol, and the clot-on-boiling test has revealed marked differences in behaviour between cultures.

2. At 37·5° C., with the exception of two cultures, there was good agreement between the time of reduction of methylene blue and resazurin. The two exceptions were a group B *Streptococcus* which reduced methylene blue but failed to reduce resazurin during the 9 hr. period of observation and an unclassified *Streptococcus* growing at 45° C. which reduced resazurin before methylene blue; the latter dye was found to retard the multiplication of the culture slightly.

3. The staphylococci, some micrococci, the *coli-aerogenes* strains and some group D streptococci were active in dye reduction at 37·5° C., while the achromobacteria, chromobacteria, Gram-positive rods, microbacteria and streptococci of groups B and E, and the two heterofermentative streptococci were inactive. Of group N the *Str. lactis* types were fairly active in dye reduction, while strains more closely related to *Str. cremoris* failed to reduce the dye.

4. At 22° C. all the cultures active in dye reduction at 37·5° C. were also active at 22° C. The achromobacteria, some micrococci and streptococci of groups B and E were also active at this temperature. All the group N strains were active at 22° C.

5. Whilst all the cultures active in milk spoilage were active in dye reduction at 22° C. many of the cultures active in dye reduction failed to reach an end-point to either the alcohol or the clot-on-boiling test within the 72 hr. period of observation. The groups N and D streptococci and the *coli-aerogenes* group were most active in milk spoilage, followed by the hetero-fermentative streptococci, and streptococci of groups B and E. The staphylococci and some micrococci were moderately active, the microbacteria were slow in reaching end-point, while other micrococci, the achromobacteria, the chromobacteria and the Gram-positive rods were inactive.

6. Studies mixing strains of *Str. lactis* with a *Staphylococcus*, orange *Micrococcus*, two strains of *coli-aerogenes* bacteria and strains of achromobacteria, and an unpigmented *Micrococcus* have not shown any effect on dye reduction at 37·5° C. or 22° C. or on milk spoilage at 22° C. due to the mixed cultures. It has shown, however, that at 37·5° C. the *Str. lactis* cultures grow more slowly than the *coli-aerogenes* bacteria, *Staphylococcus* or orange *Micrococcus*, but at 22° C. *Str. lactis* grows more rapidly than any other culture examined.

7. The results suggest an explanation for the poor relationship with some samples between the dye reduction tests at 37·5 and 22° C., and between these tests and keeping quality at 22° C. While some organisms are encouraged by a temperature of 37·5° C. and are mainly responsible for reducing the dye at this temperature, others are encouraged by a temperature of 22° C., but at this temperature organisms responsible for reducing the dye, owing to an initial numerical superiority, are not necessarily those which will ultimately cause milk spoilage.

8. With all but one culture, *Str. faecalis* 775, the end-point with the alcohol test was shortly followed by the end-point of the clot-on-boiling test.

APPENDIX

THE EFFECT OF METHYLENE BLUE ON CULTURE 32. R. 2

During the examination of the raw milk supplies (Garvie & Rowlands (10)) it was observed that while the reduction of resazurin by sample 32 occurred in 3½ hr., the reduction of methylene blue was not recorded until 5½ hr. Plates were prepared from both the resazurin and methylene-blue tubes and colonies isolated from both plates. It was found that seventeen of the twenty-two colonies isolated from the plate prepared from the resazurin tube, and seven of the twenty-one colonies isolated from the plate prepared from the methylene-blue tube were the same strain of *Streptococcus*. A representative culture, 32. R. 2, was selected and examined for the effect on dye reduction tests following the technique outlined in this paper.

On one day, samples of milk were inoculated with different volumes of the broth culture 32. R. 2. The volume of broth added to the second tube was one-fifth and to the third tube one-tenth of the volume added to the first. At the time of reduction of resazurin plate counts on yeastrel milk agar were prepared from both the resazurin and methylene-blue tubes. Incubation of both tubes in the 37·5° C. water-bath was continued, and at the time of reduction of methylene-blue plate counts were again pre-

Table 3. *Dye-reduction test, keeping quality and plate counts of* 32. *R.* 2

Volume of broth culture inoculated into 50 ml. of milk	Time of reduction				Time of end-point of the C.O.B. test	Initial count	Time of resazurin reduction						Time of methylene-blue reduction					
	37·5° C.		22° C.				Resazurin tube			Methylene-blue tube			Resazurin tube			Methylene-blue tube		
	Resazurin (hr.)	Methylene blue (hr.)	Methylene blue (hr.)				Count	Increase in count	Generation time (hr.)	Count	Increase in count	Generation time (hr.)	Count	Increase in count	Generation time (hr.)	Count	Increase in count	Generation time (hr.)
1·0	6	11	56	24		703,000	92,000,000	130·8	0·85	3,100,000	4·4	2·81	1,576,000,000	2240	0·99	118,000,000	167·7	1·48
0·2	4½	8	32	27		140,300	138,000,000	982·0	0·45	3,780,000	26·9	0·95	230,000,000	1635	0·75	18,000,000	128	1·18
0·1	4½	7	32	29		70,300	74,000,000	1061·0	0·45	1,430,000	20·3	1·04	590,000,000	8390	0·54	16,600,000	238	0·89
2·0	5	11½	+36	24		873,000	25,000,000	29·1	1·03	1,840,000	2·11	4·65	810,000,000	926	1·17	33,800,000	38·7	2·21
0·4	3½	5½	21	24		174,600	50,000,000	289·0	0·43	3,930,000	22·5	0·77	81,000,000	464	0·64	55,200,000	316	0·66
0·2	4	6	29	24		87,300	105,000,000	1205·0	0·39	3,190,000	36·7	0·77	218,000,000	2490	0·53	32,000,000	366	0·71

pared from both the methylene blue and resazurin tubes. The plates were incubated at 30° C. for 3 days. The *Streptococcus* formed long chains in milk and the plate count would give only an approximate estimate of the numbers present, but the counts served to indicate what was happening.

DISCUSSION

The results are given in Table 3. In every sample 32. R. 2 reduced resazurin before methylene blue, and therefore, the results obtained with the raw milk sample 32 are a reflexion of the behaviour of the strain of *Streptococcus* present in the milk.

The results of the plate counts show that the bacteria were increasing more rapidly in number in the tube containing resazurin than in the tube containing methylene blue, but with both dyes reduction occurred when the count was of the order of 10,000,000 to 100,000,000 organisms/ml. These results show that the multiplication of *Streptococcus* 32. R. 2 was retarded in the presence of methylene blue at the concentration used in the methylene-blue reduction test.

It would be expected that the greater the number of cells in the milk the shorter the reduction time and the keeping quality would be. The results show that while, with culture 32. R. 2, this is true of keeping quality, as measured by the clot-on-boiling test, it is untrue of the reduction tests. The tube containing the largest inoculum had the longest reduction time whether the dye present was methylene blue or resazurin, and in these tubes the generation times were considerably increased over the tubes with a smaller inoculum. Growth was, in this sample, also delayed in the resazurin tube. Whatever substance was interfering with the development of the culture it was in some way linked with the presence of the dyes, for no irregularities were observed with the keeping quality test. The peculiarities of this culture were not a reflexion of temperature, because the methylene-blue tubes incubated at 22° C. reached end-point in the same order as those incubated at 37·5° C. (i.e. 2, 3, 1). In every sample dye reduction at 22° C. occurred *after* the end-point of the keeping quality test. While with organisms producing a rennet clot in milk this is a usual phenomena, it does not occur with acid producers. Culture 32. R. 2 produces acidity in milk and these observations lend further support to the conclusion that methylene blue has a retarding effect on the development of 32. R. 2.

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481. SIMPLE AND RAPID METHODS FOR THE ESTIMATION OF BACTERIAL PHOSPHATASES USING DI-SODIUM *p*-NITROPHENYLPHOSPHATE AS SUBSTRATE

By J. TRAMER*

Co-operative Wholesale Society Ltd., Milk and Milk Products Department, London

(With 10 Figures)

In 1943, Bray & King(1) used phenolphthaleinphosphate incorporated in a solid medium to demonstrate the production of phosphatase by growing organisms. They thought that reactions depending on bacterial phosphatases had not previously been used for the identification of micro-organisms mainly because of the difficulties in technique. They considered their test to be of value in differentiating between members of certain genera, but found the slow rate of enzymic hydrolysis of phenolphthaleinphosphate to be a drawback. Hammer & Olson(2) found phosphatase production by organisms to be useful for the identification of species, and stressed the need for tests which are not too time-consuming and which have a real significance in identifying the organisms.

In work(3) on the Aschaffenburg & Mullen milk phosphatase test(4) it has been found that the substrate di-sodium *p*-nitrophenylphosphate (*p*NPP) is suitable for simple and rapid tests. *p*NPP can be incorporated in either solid or liquid media. On hydrolysis of *p*NPP by bacterial phosphatases *p*-nitrophenol is liberated: it is yellow in alkaline solutions and serves as a direct measure of enzyme activity. It will be shown that the accuracy of phosphatase estimations can be much improved by growing an organism in a suitable liquid medium and then, after a suitable period of growth, testing for phosphatase activity.

EXPERIMENTAL

The substrate

Di-sodium *p*-nitrophenylphosphate† (*p*NPP) is easily and rapidly hydrolysed by bacterial phosphatases. The salt is unstable at temperatures above 25° C. and sensitive to light. It should be kept in the refrigerator in a dark stoppered bottle. In solutions, *p*NPP is also unstable at high temperatures and can, therefore, not be sterilized by autoclaving or steaming. Seitz-filtration, however, is satisfactory.

Heat stability of buffer substrate solution

The decreases in the heat stability of M/400 substrate solutions buffered at pH 5·7 are shown in the lower curves of Figs. 5 and 6. Under these conditions the solutions appear to be reasonably stable at 37·5° C. for about 5 hr. Above 50° C. auto-hydrolysis after 1 hr. incubation is very marked, and above 60° C. it is so rapid as to invalidate experimental findings.

* Now with United Dairies Ltd., Central Laboratory, London, W. 12.
† Available from British Drug Houses Ltd., Poole, Dorset.

Test for purity of p-*nitrophenylphosphate*

A M/400 buffer substrate solution (alkaline or made alkaline) when viewed in a 25 mm. cell in a Lovibond comparator with distilled water as a blank, should give a colour not exceeding that of the permanent colour standard glass '0·1 p.p.m. chlorine', referred to later. Alternatively, the extinction coefficient in a photoelectric absorptiometer should not exceed 0·040 when compared with distilled water in a 2 cm. cell, using a violet Ilford spectrum filter with maximum transmission at about 430 mμ.

Use of p-*nitrophenylphosphate incorporated in a solid medium*

In the first experiments 1 ml. of a 1/10 dilution of milk in one-quarter strength Ringer's solution was pipetted into a Petri dish followed by 1 ml. of a sterile M/200 *p*NPP solution, buffered at pH 10 by means of the buffer used in the Aschaffenburg & Mullen milk phosphatase test (4). Ten ml. of 1·5% milk agar was added and well mixed. After 24 hr. at 37° C. there were in some cases intense yellow colonies on the plates. That the yellow colour was due to phosphatase production by micro-organisms was confirmed by subculturing the colonies into various other media and by testing for phosphatase as described later under 'Procedure A'. Experience showed that better results can be obtained with this method by using 1 ml. of a sterile unbuffered *p*NPP solution in water, instead of the buffered one. For streaking, *p*NPP plates were prepared in the following manner: 1 ml. of a sterile unbuffered M/100 *p*NPP solution in distilled water was pipetted into a Petri dish followed by 10 ml. of 1·5% milk agar and well mixed. When solid the surface was either streaked with the inoculum or inoculated from a peptone water culture by touching the surface with a straight needle. With *Bact. aerogenes*, for instance, yellow colonies were visible within 8 hr. at 37° C. After 24 hr. when the colonies were intense yellow in colour, diffusion of the *p*-nitrophenol from the colonies into the surrounding medium had occurred. Incubated controls remained unchanged, showing that there had been no auto-hydrolysis. Even quicker results can be obtained by transferring part of a colony from an agar plate with a loop to an agar plate prepared with *p*NPP as just described. *Bact. aerogenes* then produced a marked yellow colour within a few hours. These methods have been found useful as preliminary sorting tests.

Use of p-*nitrophenylphosphate in liquid media*

The medium into which *p*NPP is to be incorporated should be near or above neutrality and its pH should not vary significantly during incubation, and it should preferably be colourless. It should be borne in mind that the product of hydrolysis, *p*-nitrophenol, changes from yellow to colourless from pH 7 with increasing acidity. In addition, much auto-hydrolysis of the *p*NPP was produced when tests were carried out with acid-producing organisms in fermentable media containing *p*NPP. This made it difficult to estimate how much of the liberated *p*-nitrophenol was due to enzyme activity and how much to auto-hydrolysis.

Experiments were carried out with 10 ml. quantities of either peptone water pH 7·5 or Koser's citrate pH 6·8 which were found useful for this test. One ml. quantities of sterile, unbuffered M/200 *p*NPP solutions in distilled water were added to these media and mixed by sucking up and down in the pipette. These mixtures were then inoculated and incubated for 24 hr. at 37° C. Strong yellow colours in peptone water were obtained

with *Bact. aerogenes*, *Friedlander's bacillus* and *Proteus*, while *Bact. cloacae* and *Bact. coli* I produced weak colours. *Intermediate types Bact. coli* I gave fairly strong colour. Though turbidity made the colour estimations rather difficult, especially with the poor phosphatase producers, it was, nevertheless, possible to arrive at an approximate figure. In these and all the other experiments the tubes used were those conforming to B.S.S. 625 (1935) 152/16, made of colourless glass with rubber stoppers. Liquid media containing *p*NPP solutions are well suited for the demonstration of phosphatase production by growing organisms. In addition, this technique makes it possible to combine two tests, e.g. the citrate test and the phosphatase test. Thus, it has been found practicable to add a *p*NPP solution to Koser's citrate and to obtain two results simultaneously, e.g. *Bact. aerogenes* citrate and phosphatase positive, *Bact. cloacae* citrate positive, phosphatase negative.

Technique of colour estimations

Procedure 1: *using a comparator in transmitted light*

A Lovibond all-purpose comparator was used with the comparator disks KDA and KDAB designed for the estimation of chlorine in water by the *o*-tolidine method, for which they are calibrated in terms of p.p.m. chlorine. It was found that the colour standards of these disks give a very good match with the colours produced by the liberated *p*-nitrophenol. The standards used ranged from 0·1 to 2·0 p.p.m. chlorine. The colours were compared in transmitted light against a blank consisting either of a tube with a culture but without *p*NPP, or of a boiled culture with a *p*NPP solution, using the disks mentioned above. The colours were recorded to the nearest standard and those falling between two standards were estimated approximately.

Procedure 2: *using a comparator in reflected light*

When turbidity was present the comparison in transmitted light was difficult and it was found advisable to make the sample opaque by the addition of a measured amount of boiled milk. The readings were then taken in reflected light using the comparator as in the resazurin test and in the milk phosphatase test as described by Tramer & Wight (3). A tube containing the culture without *p*NPP and a similar amount of added milk served as blank.

Procedure 3: *using a photoelectric absorptiometer directly*

In the later part of the work the colours were measured in a Spekker absorptiometer. Test samples with a slight turbidity only were estimated against a blank prepared from a boiled culture producing practically the same turbidity as the test samples.

Procedure 4: *using a photoelectric absorptiometer after clearing*

With organisms producing a marked turbidity it was found useful to use an aluminium precipitant and to measure the clear filtrate. Details of procedures 3 and 4 are given later. Whatever the method of colour reading chosen, the readings must be taken immediately after the tests have been completed, since it has been found that the colours intensify on standing whether protected from light or not.

Phosphatase estimation of bacterial suspensions (procedure A)

Ten ml. quantities of peptone water (pH 7·5) were inoculated and incubated for 48 hr. at 37° C. 1 ml. of the suspension was transferred to the specified type of test-tubes, followed by 5 ml. of an unbuffered M/400 *p*NPP solution in distilled water and 3 drops of chloroform. The tubes were stoppered, inverted several times and incubated in a water-bath at 37° C. After 30 min. the tubes were removed, inverted and readings taken in transmitted light, using the comparator method, procedure 1. The tubes were then re-incubated for a further 90 min. when the results were similarly recorded.

Calibration of chlorine disks in terms of micrograms of p-*nitrophenol*

The permanent colour standards of the chlorine disks KDA and KDAB were calibrated in terms of μg. *p*-nitrophenol per ml. of peptone water by comparison with standards containing known quantities of *p*-nitrophenol. A definite amount (0·3–1·0 ml.) of a stock solution of *p*-nitrophenol was pipetted into a test-tube, 1 ml. of sterile peptone water

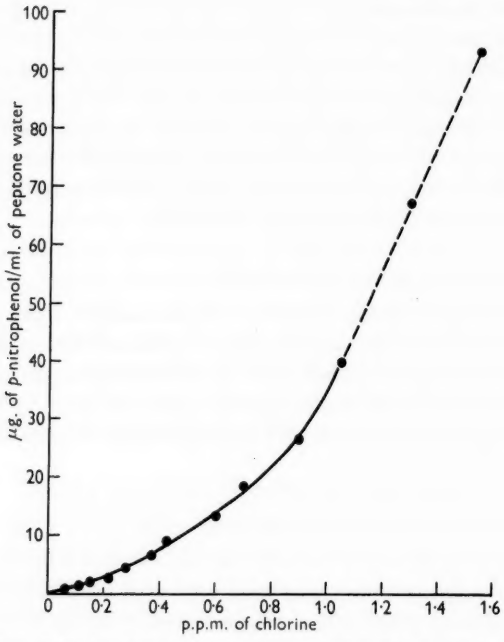

Fig. 1. Calibration of chlorine disks in terms of μg. of *p*-nitrophenol per ml. of peptone water.

added and the volume made up to 6 ml. with M/400 *p*NPP solution in distilled water. After inverting, the colour was matched in the comparator in transmitted light according to procedure 1. A tube containing 5 ml. of M/400 *p*NPP solution in distilled water and 1 ml. of peptone water served as a standard blank. The colours developed in the tubes were compared with the permanent colour standards, and values falling between two standards were estimated. The procedure was repeated on several occasions and the average reading of two workers recorded. From the results obtained a curve (Fig. 1) was

derived from which readings in 'p.p.m. chlorine' may be transformed accurately into μg. of *p*-nitrophenol per ml., when using procedure A.

It appears that under these conditions 1 μg. of *p*-nitrophenol per ml. is clearly detectable. The colour match is very good up to 1·25 p.p.m., but at higher concentrations it is less precise.

A possible application of procedure A for the differentiation of coli-aerogenes *organisms*

Leahy, Sandholzer & Woodside (5) studied the phosphatase produced by three strains of *Bact. aerogenes* and two of *Bact. cloacae* grown in sterile milk. Disodium-phenyl-phosphate was used as substrate and di-bromo-quinonechloroimide (BQC) as phenol indicator. They found that *Bact. aerogenes* was a strong phosphatase producer whilst

Table 1

M.W.B. results			Chlorine disk readings p.p.m. Peptone water cultures 48 hr. old		Counts on 48-hr.-old peptone water (millions/ml.)
No.	Gelatine liquefaction in days	Reaction at 44° C.	30 min.	2 hr.	
C. 62	3	—	N.	Tr.	—
C. 63	4	—	N.	Tr.	—
C. 28	5	—	N.	Tr.	52
C. 29	5	—	N.	Tr.	55
C. 36	7	—	0·38	0·80	—
MC 3 A	8	—	N.	N.	3
C. 35	10	—	0·37	0·80	—
MC 9 A	10	—	N.	N.	—
C. 34	13	—	N.	Tr.	—
C. 60	13	I.G.L.	Tr.	0·20	—
C. 71	13	I.G.L.	0·12	0·27	—
C. 74	13	I.G.L.	Tr.	0·18	—
MC 4	14	—	0·1	0·25	—
C. 75	16	—	Tr.	0·16	—
C. 30	23	—	0·30	0·62	—
C. 32	27	—	0·65	1·50	—
C. 73	28	—	0·21	0·53	—
C. 38	Neg.	—	0·27	0·75	23
C. 39	Neg.	—	0·42	1·0	61
C. 65	Neg.	I.	0·30	0·70	—
C. 66	Neg.	G.L.	0·42	0·95	—
C. 67	Neg.	I.G.L.	0·25	0·60	106
C. 69	Neg.	I.G.L.	0·45	1·0	—
		Irregular VI group, 44° positive			
C. 61	10	G.L.	0·30	0·65	—
MC 5	13	G.L.	0·90	1·60	—
C. 37	Neg.	G.L.	0·62	1·40	—
C. 40	Neg.	G.L.	0·32	0·85	—
C. 41	Neg.	G.L.	0·38	0·95	—
MC 6 F	Neg.	G.L.	0·70	1·50	—

G.L. = gas from lactose at 44° C. I. = + at 44° C. (indole). N. = no appreciable colour. Tr. = trace colour.

Bact. cloacae was not. Pett & Wynne (6) have shown pH 5·6 $\pm$ 0·2 to be the optimum for the phosphatase produced by *Bact. aerogenes* when using β-glycerophosphate or hexosephosphate as substrates and pH 6·2 $\pm$ 0·2 with pyrophosphate as substrate. After a few preliminary experiments with *p*NPP it appeared that the phosphatase reaction might be useful for the differentiation between these two species, particularly, since their differentiation depends at present on the gelatine liquefaction test which is time-consuming and uncertain. The Metropolitan Water Board (M.W.B.) kindly supplied twenty-nine cultures of methyl red negative, Voges-Proskauer positive, *coli-aerogenes* organisms

with information about their gelatine liquefaction times. These organisms were tested for phosphatase by procedure A, and the colours estimated by procedure 1. These tests were carried out under suboptimal conditions and the results are therefore only reasonably accurate. The optimal conditions for hydrolysis of *p*NPP by the phosphatase of *Bact. aerogenes* were subsequently established and a method is given later for more accurate assessments. The results for the twenty-nine cultures are shown in Table 1. Since these twenty-nine cultures were stock cultures, further experiments were carried out with seventeen freshly isolated, subcultured once only. They were MR negative, VP positive *coli-aerogenes* organisms also supplied by the M.W.B. The results obtained with these seventeen cultures grown for 48 hr. at 30° C. and those previously obtained with the twenty-nine cultures are combined in Tables 2A and 2B. For this purpose the disk readings were transformed into μg. *p*-nitrophenol per ml. peptone water by using Fig. 1. The organisms were divided into 44° C. negative organisms and 44° C. positive organisms (irregular VI).

Table 2A

(MR negative, VP positive, 44° C. negative, *coli-aerogenes* organisms.)

Gelatine(7) liquefaction	No. of strains	μg. of *p*-nitrophenol/ml. peptone water				
		Nil-trace	1–3	3–5·5	6–10	>10
Within 7 days	8	4	2	1	—	1
7–21 days	13	3	5	4	—	1
>21 days	14	—	2	—	—	12
Totals	35	7	9	5	—	14

Table 2B

(MR negative, VP positive, 44° C. positive *coli-aerogenes* organisms (Irregular VI).)

Gelatine(7) liquefaction	No. of strains	μg. of *p*-nitrophenol/ml. peptone water				
		Nil-trace	1–3	3–5·5	6–10	>10
Within 7 days	Nil	—	—	—	—	—
7–21 days	2	—	—	—	—	2
>21 days	9	—	—	—	—	9
Totals	11	—	—	—	—	11

Table 2A (44° C. negative organisms) shows that (*a*) of twenty-one organisms liquefying gelatine within 21 days, nineteen were found to produce little or no phosphatase; (*b*) of fourteen organisms liquefying gelatine in more than 21 days or not liquefying within 56 days, twelve were found to be strong phosphatase producers. Table 2B (44° C. positive organisms) shows that all eleven organisms were strong phosphatase producers irrespective of their gelatine liquefaction time. Thus, there is a definite relationship between gelatine liquefaction time and phosphatase production of 44° C. negative organisms only.

DETERMINATION OF OPTIMAL CONDITIONS FOR HYDROLYSIS OF *p*-NITROPHENYL-PHOSPHATE BY THE PHOSPHATASE OF SELECTED SPECIES OF *BACT. AEROGENES*

Determination of optimum pH

Three typical organisms were chosen for this determination: *Bact. aerogenes*, C. 39, a gelatine non-liquefier, 44° C. negative; *Bact. cloacae*, C. 62, a rapid gelatine liquefier, 44° C. negative; and *Irregular VI*, C. 40, a gelatine non-liquefier, 44° C. positive. C. 39 and C. 62 were grown in 100 ml. quantities of peptone water pH 7·5 for 48 hr. at 30° C.

and C. 40 was grown for 24 hr. at 44° C. Before testing, the cultures were filtered through a sterile Whatman no. 30 filter-paper to obtain a practically homogeneous suspension.

Clark and Lubbs's buffers were used. They consisted of 0·1 M-potassium hydrogen phthalate and 0·1 M-potassium phosphate solutions made up with 0·1 N-NaOH and boiled distilled water to pH values of 3·93, 4·52, 5·12, 5·72, 6·24, 6·82 and 7·90 as determined with a Cambridge pH meter. The concentration of the substrate was M/400, i.e. 0·075 g. of *p*NPP were dissolved in and made up to 100 ml. with the appropriate buffer.

To 5 ml. of the buffer substrate solution in a test-tube was added 1 ml. of the filtered suspension, followed by three drops of chloroform. The test tubes were stoppered with rubber bungs previously cleaned in boiling water, inverted several times and incubated in a 37° C. water-bath. After 1 hr. the tubes were removed from the bath, cooled in ice-water and made alkaline by adding to each tube a total of 0·3 ml. of an appropriate amount of normal caustic soda in distilled water. The quantities necessary were found earlier by titration against phenolphthalein.

Colour reading

In these experiments the yellow colour developed was measured immediately in a 'Spekker' photoelectric absorptiometer using a violet Ilford spectrum filter with a maximum transmission at about 430 mμ. 1 ml. of a boiled culture with 5 ml. of a buffer substrate solution which had been made alkaline, was used as a blank. As the colours

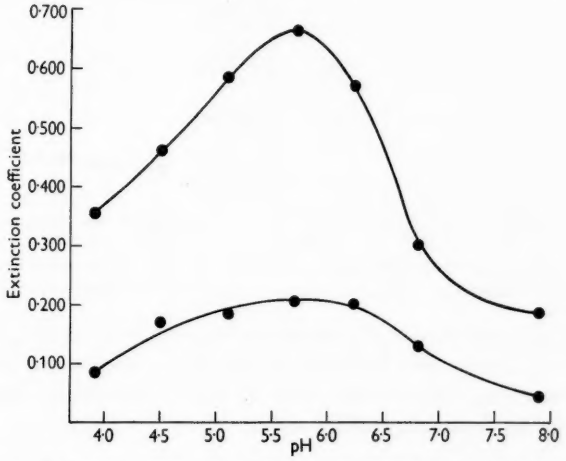

Fig. 2. Determination of optimum pH for the hydrolysis of *p*NPP by the phosphatases of *Bact. aerogenes* (upper curve) and of *Irregular VI* (lower curve).

developed were rather strong, the size of the cell chosen was 0·5 cm. and the readings obtained were therefore doubled in order to arrive at the standard extinction coefficient. The mean duplicate readings are plotted in Fig. 2. The point at pH 6·24 on the curve for *Irregular VI* is based on one result only, since the duplicate tube gave an erroneously high reading. It would appear from Fig. 2 that the optimum pH for the hydrolysis of *p*NPP by the phosphatase of *Bact. aerogenes* is about pH 5·7 and that that of *Irregular VI* seems to be in the same region. *Bact. cloacae* and the controls gave no measurable colours in the 0·5 cm. cells. The controls consisted of 1 ml. of sterile peptone water with 5 ml. of

the various buffer substrate solutions and three drops of chloroform. They were treated as the test samples but measured against an unincubated blank consisting of 1 ml. of sterile peptone water and 5 ml. of buffer substrate solution, made alkaline. Plate counts were done at the time of testing using milk agar and the results obtained for the filtered suspension were:

Bact. aerogenes, C. 39, 191×10^6, 218×10^6;
Bact. cloacae, C. 62, 246×10^6, 249×10^6;
Irregular VI, C. 40, 30×10^6, 31×10^6.

The relationship between substrate concentration and enzyme activity

The concentrations tried in this experiment were M/200, M/400, M/600, and M/800 and the culture used was *Bact. aerogenes*, C. 39, grown for 48 hr. at 30° C. in peptone water pH 7·5. The tests were carried out at pH 5·7 and hydrolysis was allowed to proceed for

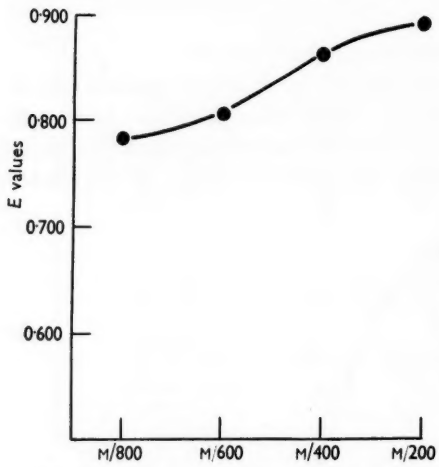

Fig. 3. Determination of optimum substrate concentration.

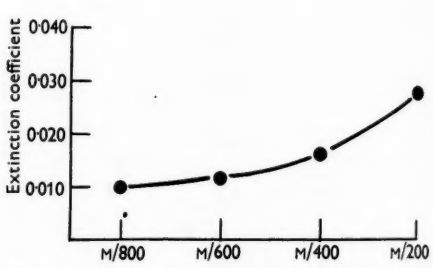

Fig. 4. *E*-values of various concentrations of buffer substrate solutions.

1 hr. at 37° C. Fig. 3 shows that the substrate concentration M/200 gives the highest readings, but it can also be seen from Fig. 4 that this concentration has a slight, but measurable, yellow colour when freshly made up and made alkaline. The concentration of M/400 was therefore chosen for further work.

The effect of various incubation temperatures on the rate of hydrolysis

Fig. 5 shows that the highest rate of hydrolysis occurs at 48° C., when testing at pH 5·7, using a substrate concentration of M/400 and an incubation time of 1 hr. However, the buffer substrate solutions appear to become considerably less stable with rising incubation temperature, as can be seen from the lower curve in Fig. 5. Experiments at 63° C. were discontinued because of the very considerable auto-hydrolysis. Although this meant a slight reduction in the rate of hydrolysis, it was decided to use the incubation temperature of 37° C. rather than that of 48° C., because the buffer substrate solution is much more stable at that temperature and the 37° C. bath is standard equipment.

The effect of incubation time on the rate of hydrolysis

For this determination a 48 hr. old culture of *Bact. aerogenes*, C. 39, was diluted with sterile peptone water 1 : 1 because the undiluted culture would have given a yellow colour too deep for accurate measurement in the Spekker absorptiometer. The pH was 5·7, the substrate concentration M/400 and the incubation temperature 37° C. Six duplicate tubes were set up containing 1 ml. of culture and 5 ml. of buffer substrate solution with three drops of chloroform. After a specified time a pair was removed from

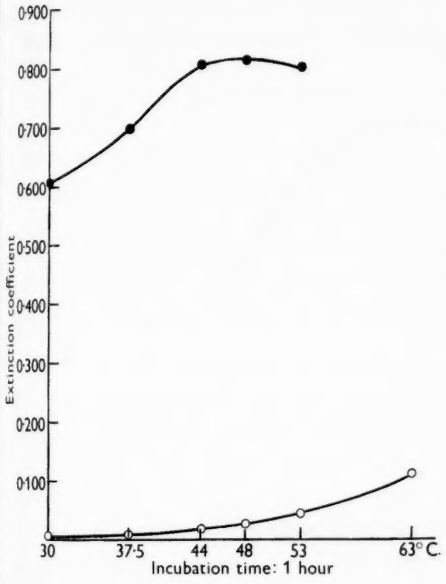

Fig. 5.

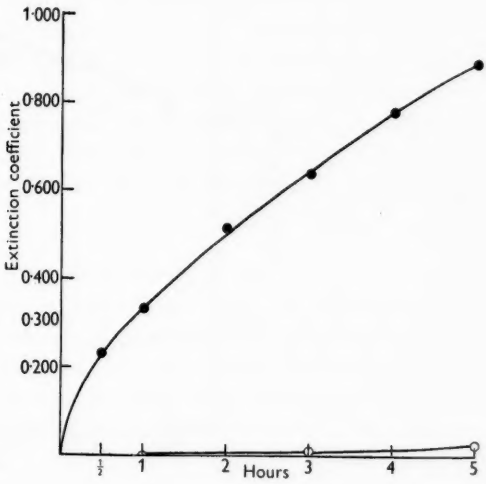

Fig. 6.

Fig. 5. Determination of optimum incubation temperature for the hydrolysis of *p*NPP by *Bact. aerogenes* and heat stability of the buffer substrate solution.

Fig. 6. Determination of the rate of hydrolysis of *p*NPP (M/400, pH 5·7) by the phosphatase of *Bact. aerogenes*. Incubation temperature 37° C.

the water-bath, made alkaline and the colour was measured photoelectrically as described under 'colour reading'. Auto-hydrolysis was determined by using peptone water with buffer substrate solutions and the colours were measured in 2 cm. cells. It can be seen from Fig. 6 that up to 5 hr. incubation the rate of hydrolysis remains fairly constant. The buffer substrate solution appeared to be quite stable up to 3 hr. and even at the end of the 5 hr. incubation time no really marked auto-hydrolysis had occurred. Since the colours developed after 1 hr. incubation were already very distinct and conveniently measurable, it was decided to limit the incubation time to this period. After that time, the colours developed were also well suited for estimation in the comparator.

THE HEAT STABILITY OF THE PHOSPHATASE OF A SPECIFIC STRAIN OF *ACHROMOBACTER* ISOLATED FROM MILK

A phosphatase-producing organism was isolated from milk by picking a yellow colony from a milk agar plate containing *p*NPP. This organism was subcultured and tested for phosphatase by the improved procedure A 1:

1 ml. of the suspension, filtered through a Whatman no. 30 filter-paper, is added to 5 ml. of an unbuffered *p*NPP solution in distilled water, followed by two drops of chloroform. The sample is incubated for 1 hr. at 37° C. and 0·1 ml. of N-NaOH is then added. This addition is made in order to develop the full colour intensity of the liberated *p*-nitrophenol and to prevent any appreciable increase in hydrolysis before the readings are taken.

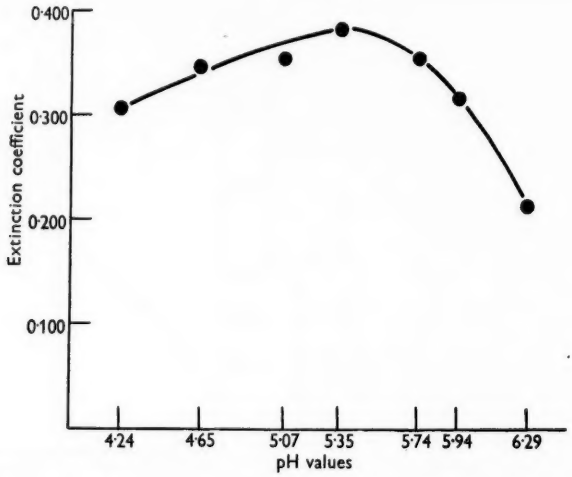

Fig. 7. Determination of optimum pH for the hydrolysis of *p*NPP by *Achromobacter*.

This procedure is recommended in cases where the optimum pH has not been established, since experience has shown that wrongly buffered *p*NPP solutions are more likely to result in false negative tests, where unbuffered *p*NPP solutions generally produce evidence of the presence of the enzyme.

The bacterial phosphatase obtained from this organism was an unusual one as it was destroyed by laboratory pasteurization at 145° F. for 35 min. The organism was therefore identified and found to be a species of *Achromobacter*. Its optimum pH for hydrolysis of *p*NPP was established using a 48 hr. old peptone water culture, grown at 30° C. and is near pH 5·35 (see Fig. 7). This culture was filtered through a Whatman no. 1 filter-paper and then tested for phosphatase production at various pH values. The buffers used in this case consisted of 0·1 M-potassium hydrogen phthalate with 0·1 N-NaOH and boiled distilled water. The range was narrowed after the approximate optimum pH was first determined. Tests were made at pH values of 4·24, 4·65, 5·07, 5·35, 5·74, 5·94 and 6·29. The colours developed were measured photoelectrically, but this time 1 ml. of a boiled culture was incubated with each buffer substrate solution and each test sample was read against its corresponding boiled and incubated blank. Though there was some turbidity,

it is believed that this effect was eliminated by using the blank. However, it has been found possible to remove the turbidity just prior to the colour reading by using aluminium sulphate and the following method, procedure 4, of colour estimation, has been adopted in the determination of the heat stability of this phosphatase, thus:

Achromobacter was grown for 6 days in 100 ml. of peptone water pH 7·5 at 30° C. The culture was filtered through a Whatman no. 1 filter-paper and 5 ml. quantities of the filtrate were pipetted into test-tubes. These were stoppered and kept in a 145° F. water-bath for 5, 7½, 10, 15 and 25 min. respectively. After that time the tubes were removed and cooled in ice water.

To duplicate tubes, each containing 10 ml. of a M/400 buffer substrate solution pH 5·3, were added 2 ml. quantities of the heat-treated cultures; tc one pair was added 2 ml. of the untreated filtrate and to another pair 2 ml. of the filtered and boiled culture to serve as blank. Three drops of chloroform were added to the tubes which were stoppered, inverted and incubated for 1 hr. at 37° C. After that time the tubes were removed from the water bath, 0·6 ml. of N-NaOH was added, followed by 1·0 ml. of a 5% w/v solution of aluminium sulphate in water. The contents were shaken and filtered through a Whatman no. 30 filter-paper. 10 ml. of the clear filtrate were collected in a graduated test-tube, 0·5 ml. of N-NaOH added and the colours read in the 'Spekker' absorptiometer against the blank which had been treated as the rest. The *E* values converted into μg. *p*-nitrophenol as described below, are given in Fig. 9.

Conversion of E *values into* μg. *of* p-*nitrophenol per ml.*

A stock solution of *p*-nitrophenol was prepared, and accurate amounts pipetted into test-tubes containing 2 ml. of a boiled culture of *Achromobacter* and *Bact. aerogenes*, C. 39, respectively. The volume was made up to 12 ml. with the appropriate buffer substrate solutions and the procedure was the same as in the actual test, using procedure 4 for the colour estimation. The blank used consisted of the buffer substrate solution and the boiled culture only, and was treated as before. The results are given in Fig. 8, which makes it possible to transform absorptiometer readings into μg. of *p*-nitrophenol/ml.

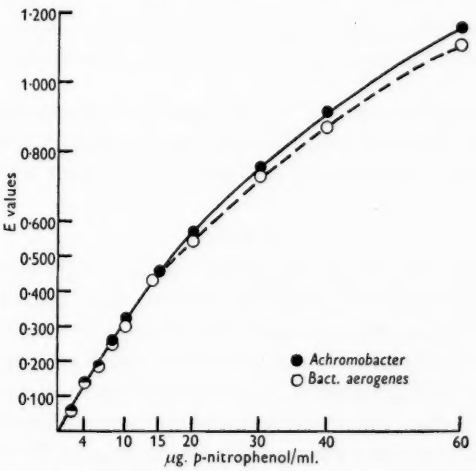

Fig. 8. Conversion of *E* values in μg. *p*-nitrophenol/ml.

Comparison between the heat labile phosphatase produced by a strain of Achromobacter *and that of* Bact. aerogenes *C.* 39

It was of interest to make a comparison between these two bacterial phosphatases, and for this purpose a 3-day-old culture of *Bact. aerogenes*, C. 39, in peptone water was tested for phosphatase using the same technique as for *Achromobacter* (Fig. 10).

Fig. 9 shows that the *Achromobacter* produced a phosphatase with a heat stability very similar to that of milk phosphatase, being inactivated at 145° F. in less than 30 min. The phosphatase of *Bact. aerogenes*, C. 39 (Fig. 10), on the other hand, is much more

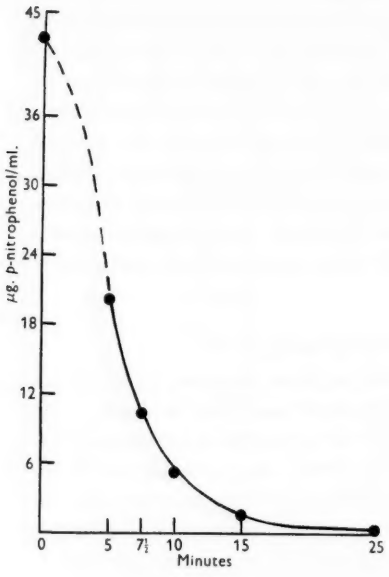

Fig. 9.

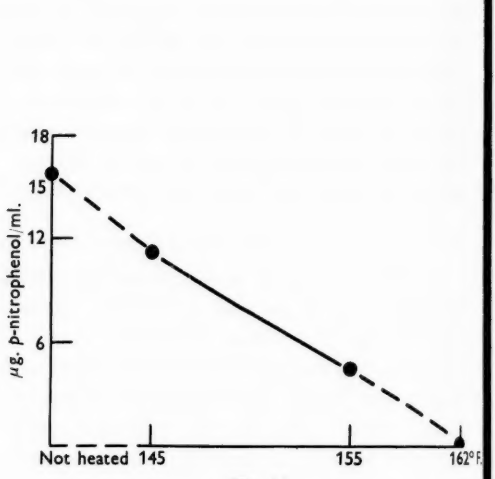

Fig. 10.

Fig. 9. The heat stability of the phosphatase produced by *Achromobacter*. Incubation temperature 145° F.

Fig. 10. The heat stability of the phosphatase produced by *Bact. aerogenes*. Holding time 40 min.

heat-resistant, heat treatment at 145° F. for 40 min. reducing its phosphatase content by only about one-third. A similar reduction had also been found on two previous occasions under slightly different conditions of test and with stronger initial concentrations of phosphatase.

SUMMARY

Bacterial phosphatases of growing organisms can be simply demonstrated by incorporating di-sodium-*p*-nitrophenylphosphate (*p*NPP) into suitable media. The phosphatase content of a bacterial suspension can be assessed by incubating a measured amount of it with unbuffered or suitably buffered *p*NPP solutions. The yellow colour (in alkaline solution) of *p*-nitrophenol liberated from the substrate by phosphatase can be measured accurately and conveniently in a photoelectric absorptiometer, or estimated in a Lovibond comparator using permanent colour standards. The usefulness of the tests for differentiating between members of certain genera is demonstrated by the reactions

of *Bact. aerogenes* and *Bact. cloacae*, for which a close relationship between phosphatase production and gelatine liquefaction times has been found. The optimal conditions for hydrolysis of *p*NPP by the phosphatase of a strain of *Bact. aerogenes* have been established.

A heat-labile phosphatase has been found to be produced in peptone water by a strain of *Achromobacter*, isolated from milk. Its optimum pH for the hydrolysis of *p*NPP was determined and its low heat resistance contrasted to that of the phosphatase produced by *Bact. aerogenes*, C. 39, which was considerably more heat stable.

The properties of the substrate and of its solutions are discussed and various methods of colour estimation are described.

The author wishes to thank Dr A. T. R. Mattick and Dr R. Aschaffenburg for their criticisms and advice, Mr N. P. Burman from the Metropolitan Water Board for the provision of the cultures and Mr J. R. Donaldson for his assistance in the early part of the work. My thanks are due to the Directors of C.W.S. Ltd., for permission to publish this paper and to the Directors of United Dairies Ltd., for facilities to carry out the final experiments.

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482. 'WILD' STRAINS OF *STREPTOCOCCUS CREMORIS*

By A. HIRSCH

National Institute for Research in Dairying, University of Reading

Streptococcus cremoris and *Str. lactis* are widely used as starters in cheese-making, and the differentiation of these two organisms is of some importance. Many workers regard the two organisms as separate species, but the more cautious (1) consider that *Str. cremoris* is a mere environmental variety of *Str. lactis*. Yawger & Sherman (2) recovered *Str. cremoris* from raw-milk supplies which were unlikely to have been contaminated with laboratory strains. It was also recovered from bulk milk by Abd-el-Malek & Gibson (3).

When considering the question of the occurrence of *Str. cremoris* in the wild state, it may be an objection that Yawger & Sherman and Abd-el-Malek & Gibson were working in America and England respectively, where cultivated starters containing the organism are in common use. During a recent visit to the Jura Mountains in France, the opportunity was taken to isolate lactic streptococci from local milk supplies. Two of them proved to be *Str. cremoris*, and it appears very likely that this organism does in fact occur in the wild state.

MATERIAL AND METHODS

Materials. Strains of lactic streptococci were isolated from the milk of four producers in the Jura (France) Mountains. The four producers were about 80 miles apart, and they were at an average altitude of *c.* 2000 ft. None had been in previous contact with a laboratory producing streptococcal starters, and all used rather primitive methods of milk production.

Methods. A loopful of the milks was streaked on Yeastrel-glucose agar containing thallous acetate (1/2000) and incubated at 30° C. for 2 days. Single colonies were then picked into litmus milk.This culture was again streaked on milk-agar and single colonies picked into litmus milk.

The cultures were further classified by Sherman's tests (4). Tests for the production of ammonia from arginine and the fermentation of maltose and dextrin, which are additional distinguishing tests, were also made (5,6). The organisms were also grouped serologically by Dr C. A. E. Briggs, using sera prepared by himself.

RESULTS

All the thirty-five strains isolated reacted only with group N serum. Two of the strains could be classified with certainty as *Str. cremoris* (Table 1).

Table 1. *The cultural and biochemical reactions of two group N streptococci isolated from milk produced in the Jura Mountains*

		Physical resistance tests									
		Growth		Sugar fermentations					NH_3 produced		
Species	Strain no.	In 4% NaCl	At 40° C.	Mannitol	Sucrose	Raffinose	Maltose	Dextrin	from arginine	Growth controls	
Str. cremoris	19/1	–	–	–	–	–	–	–	–	+ + +	
Str. cremoris	22	–	–	–	–	–	–	–	–	+ + +	

SUMMARY

Of thirty-five strains of streptococci isolated from milk produced in the Jura mountains, two were strains of *Streptococcus cremoris*. It appears, therefore, that this organism occurs in the wild state.

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483. THE EVOLUTION OF THE LACTIC STREPTOCOCCI

By A. HIRSCH

National Institute for Research in Dairying, University of Reading

Although milk may be considered to be the normal habitat of the lactic streptococci, their adaptation to this environment must have followed the domestication of mammals, for these organisms are not pathogenic and do not normally occur in the udder. If this reasoning is accepted, then the small biological age of these organisms logically follows, and possibly they are the youngest members of this genus.

The hypothesis that the lactic streptococci are of recent origin gains support from several lines of reasoning, when the large body of information which has been accumulated for this economically important group of micro-organisms is examined.

(1) The evidence of the saprophytic nature of the lactic streptococci

According to Zinsser (1), pathogenic organisms may be regarded as unsuccessful parasites. They are in an unbalanced state with the host and either the host or the invader is killed. As the parasitism becomes perfected, so a *modus vivendi* is arrived at between the host and the parasite. Such specialization requires time, and it is likely that biologically the younger organism is the saprophyte rather than the parasite.

Most species of the streptococci are pathogenic, or potentially so, and almost all are parasitic. The lactic streptococci form a noteworthy exception and they have never been shown to be pathogenic. Only Wagner (2) claimed to have isolated these organisms from two diseased persons, but other reports to this effect employing modern diagnostic methods do not seem to exist. In addition, many millions of these organisms must be consumed daily without ill effects. The optimum temperature, 30° C., of growth is also unfavourable for a pathogen. *Str. cremoris*, in particular, grows poorly or not at all at body temperature (see also Sherman's review (3)).

The non-parasitic nature of these streptococci therefore supports the supposition that biologically they are young. It is, of course, not argued that all saprophytic organisms are young, but in this case, when the age of organisms within the same genus is under consideration, this is additional deductive evidence. As these organisms are primarily saccharolytic, and they are best known for their milk-souring activities, it is pertinent to inquire whether their power to ferment lactose is a constant property.

(2) The evidence of the lactose fermentation of the lactic streptococci

Yawger & Sherman (4) described in 1937 lactose non-fermenting strains of *Str. lactis*. A quite distinct problem, and one which concerns this discussion much more intimately, is the ease with which 'normal' strains of the lactic streptococci lose their ability to ferment lactose. A case in point is the 'weakened' strains described by Davis (5). These were isolated from cheese when the lactose present had already been utilized. The cultures had lost their ability to ferment lactose, but without impairment of the glucose fermentation. Hunter (6), studying the colony forms of *Str. cremoris*, has shown that his 'weakened'

strains became rougher as the ability to ferment lactose was lost. Hirsch(7) reported that, after growth in a glucose medium, the inhibitory *Str. lactis* used lost its ability to ferment lactose, and this could not be recovered by repeated subculturing in milk. In addition, it would appear that other fermentative properties of *Str. lactis* are more constant than its lactose fermentation. Thus Sherman & Hussong(8) isolated 757 substrains of maltose-positive, sucrose-negative strains. None of their substrains changed to maltose-negative and only one substrain became sucrose-positive.

Thus some properties of *Str. lactis*, which may have no natural function, appear to be less variable than its ability to sour milk—a vital fermentation. This is additional evidence that adaptation to milk, which is a new habitat, is taking place now.

(3) THE EVIDENCE OF THE HABITAT OF THE LACTIC STREPTOCOCCI

Much of the early work on the ecology of the lactic streptococci had little value, because reliable methods of distinguishing these organisms from the faecal streptococci did not exist. More recently Stark & Sherman(9) succeeded in isolating strains of *Str. lactis* from vegetable sources, but they were unable to come to any definite conclusions because their organisms occurred only in small numbers. Bång(10) claimed that *Str. lactis* could establish itself and even multiply in the pig gut when the animals were fed on milk. In recent work from this Institute, this claim was not substantiated(11).

The occurrence of antibiotic-producing strains of *Str. lactis* was first surveyed by Cox & Whitehead(12), who found them in silage, coats and udders of cows and in dung. Hirsch & Wheater(13) isolated these organisms from a number of sources, including human throats and faeces.

It appears from this evidence that the habitat of these micro-organisms is still uncertain and that they occur ubiquitously in small numbers. In laboratory practice, however, raw milk is used as an infallible source of the lactic streptococci. In raw milk which is stored at atmospheric temperature the lactic streptococci are the predominating flora, as was shown by Rowlands, Barkworth, Hosking & Kempthorne(14), who studied the reasons for the discrepancy in the results of keeping quality tests done at 37 and 18° C. These organisms predominated in enzymatic activity and numbers, a fact which is difficult to explain if a habitat other than milk is postulated. It would appear possible, and the evidence cited does not contradict it, that milk in its 'man-made' form is fast becoming, or is already the true ecological source of the lactic streptococci and, for a situation like this, there are numerous biological similes.

The data adduced and their logical connexion afford strong proof for the evolutionary hypothesis of this paper, but even further support is furnished by the production of antibiotics by these organisms.

(4) THE EVIDENCE OF THE ANTIBIOTIC PRODUCTION OF THE LACTIC STREPTOCOCCI

Some authors have searched for a biological explanation for the occurrence of antibiotics. Such search was often in vain because these antibiotics may represent a still surviving but now useless function, or they may be the result of chance mutations. In the lactic group of organisms, however, the two recognized species each produce distinct antibiotics directed primarily against the other lactic organism, so that in this group the production of antibiotics has the apparent function of ensuring preponderance by inhibiting the keenest competitor.

Before further discussing this point, it is necessary to define further the two species constituting the lactic streptococci or serological group N (15).

Str. cremoris was defined as a new species in 1919 by Orla-Jensen (16), and this distinction has been accepted by Bergey (17). Recent investigations have further confirmed this distinction. Thus Yawger & Sherman (18) have isolated strains from raw milk and distinguished them from *Str. lactis* by the inability of *Str. cremoris* to grow at pH 9·2, to grow at 40° C., or to grow in broth with 4% added salt. Later the same group of investigators found that the production of ammonia from arginine was a further useful differential criterion (19). Hunter added to the list of criteria the fermentation of maltose and dextrin (20), and, in a survey of streptococci from milk, Abd-el-Malek & Gibson broadly confirmed these findings (21). The evidence of the phage typing can also be mentioned. Hunter (20) showed that not only are the organisms attacked by different phages, but that the 'phage patterns' were also different. Working independently of Hunter, Nichols & Hoyle (22) confirmed his results. Moreover, recently both organisms were isolated from a virgin source, so that there can be little doubt that both species occur in the 'wild' state (23).

Oxford (24) purified and described the antibiotic diplococcin, which is produced by *Str. cremoris*. Mattick & Hirsch (25) distinguished it from nisin, the antibiotic produced by *Str. lactis*, in a number of ways, but their main differentiating character was in the mutual inhibition of the antibiotic-producing organisms. These observations were eventually confirmed and extended (26,27).

Nisin is inhibitory to a varying extent to most Gram-positive organisms (25), but the most sensitive organism to nisin is *Str. cremoris* (see Table 1). The antibiotic diplococcin is even more specific and is inhibitory only against *Str. lactis*, with this test organism, however, high titres of 1/320 can frequently be obtained in dilution assays. Some of these results are shown in Table 1. Several saprophytes were used as test organisms.

Table 1. *The effect of two inhibitory streptococci on certain Gram-positive non-pathogenic bacteria*

	Test organisms							
	Antibiotic producers		Other cultures					
Antibiotic produced by	*Str. lactis* (*x*)	*Str. cremoris* (*y*)	*Str. lactis*	*Str. cremoris*	*Str. thermophilus*	*Str. faecalis*	*Leuconostoc*	*Lactobacillus*
Str. lactis (*x*)	<1/10	1/2560	1/20	1/2560	1/10	1/10	1/120	1/120
Str. cremoris (*y*)	1/160	<1/10	1/320	1/10	<1/10	<1/10	<1/10	<1/10

The figures are averages (with several strains of test organisms) of dilutions at which growth of the test organism is still inhibited when the growth controls appeared fully grown.

These high inhibitory titres can only be obtained with the culture fluids of the lactic streptococci (13). Almost 10,000 samples of material rich in streptococci were examined, and whenever an inhibitory *Streptococcus* with a high titre was isolated, this was identified as belonging to the lactic streptococci. This interesting observation further supports the hypothesis that milk is now in the process of, or has already become, the habitat of these organisms, and that these antibiotics are therefore the expression of the struggle for existence.

Orla-Jensen (28) has suggested *Str. faecalis* as the parent organism of *Str. lactis*, and the similar behaviour of these two organisms supports such a hypothesis. It would appear less likely that *Str. cremoris* originates from the same organism. The strong mutual inhibition of *Str. lactis* and *Str. cremoris* becomes more understandable when adaptation to the same environment of two distinct organisms is considered. The low temperature of growth and other characters of *Str. cremoris* suggest a relationship more akin to those streptococci occurring on plants, e.g. *Str. diacetilactis* (29) and the pediococci (30).

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484. STUDIES IN THE BACTERIOLOGY OF MILK

IV. THE GRAM-NEGATIVE RODS OF MILK

By Y. ABD-EL-MALEK* and T. GIBSON

Bacteriology Department, College of Agriculture, Edinburgh

Previous papers of this series(1,2,3) discussed the streptococci, staphylococci, micrococci and corynebacteria that were isolated from quantitative platings of various types of raw and pasteurized milk. The method of isolation employed was purposely non-selective, and the organisms detected may therefore, in most cases, be regarded as having been among the dominant bacteria in the samples. The Gram-negative rods that were isolated were grouped as in Table 1, which also shows the type of milk from which they were obtained. Samples taken by aseptic milking are not included in the table, since in them Gram-negative bacteria were not detected. The two species of *Alcaligenes* may give an equivocal reaction in Gram's method of staining, but they appear to be closely related to the Gram-negative series, and it is convenient to place them there.

Table 1. *Classification and sources of the cultures examined*

	Raw milk	Pasteurized milk when fresh	Pasteurized milk when tainted
No. of samples examined	23	54	28
No. of samples yielding:			
Alcaligenes viscosus	19	0	0
Alcaligenes tolerans n.sp.	6	6	17
Coli-aerogenes group	6	0	0
Fluorescent pseudomonads	12	0	0
Other Gram-negative rods	12	0	0

The last two groups mentioned in the table comprise organisms that we have detected, not only in raw milk, but also on wet unsterile dairy equipment where they dominated the bacterial population. They were not examined in detail and were not closely identified, but some of their characters were determined. None of them formed acid in milk or lactose peptone water. The fluorescent types all brought about an active hydrolysis of casein and fat. In the series described in the table as 'Other Gram-negative rods' three main types were distinguished:

(*a*) Motile organisms which form unpigmented or yellow to orange colonies. Although the formation of soluble pigments was not detected, they possibly belong to *Pseudomonas*, since polar flagellation was proved for some of them. Their action on casein varies from none to rapid, as does their lipolytic activity.

(*b*) A non-motile chain-forming *Flavobacterium* which forms yellow to orange-coloured colonies. It is actively caseolytic and not lipolytic. This organism is similar to a type found in dairy cooling water by Elis Jones & Thomas(4).

(*c*) Non-motile *Achromobacter* types which are variable in action on casein and fat.

* Present address: Faculty of Agriculture, Fouad I University, Giza, Egypt.

The organisms identified as *Alcaligenes* were of particular interest on account of the frequency with which they were detected. Problems arose concerning their specific identity and characterization, and these organisms were made the subjects of special study. The remainder of the present paper is concerned with that phase of the work.

Alcaligenes

This generic name is here employed for a group of milk bacteria possessing the following combination of characters:

Cells rod-shaped to coccal, in dried films usually 0·7–1 μ in diameter, at times up to 2 μ, often in pairs and short chains, non-motile, Gram-negative or -variable.

Growth on solid media often viscid, unpigmented or (only in old cultures) slightly brown.

No acid formed from sugars.

No proteolytic action on gelatin or casein.

Litmus milk usually alkaline, rarely unchanged in reaction; may be ropy.

Alcaligenes is widely regarded (see especially Conn (5)) as a poorly defined genus, and the milk organisms probably differ from its type species to a greater degree than do the species that were transferred from it to *Agrobacterium* by Conn. In the meantime, however, there seems to be no satisfactory alternative to retaining in *Alcaligenes* the types that occur in milk.

Interest in these organisms in relation to milk has been largely concentrated on their ability to produce a ropy condition. They neither form acid nor attack protein, thus suggesting that their importance is restricted to occasional outbreaks of ropiness. Our work indicates that this is an incomplete picture. The organisms have been found among the dominant bacteria in raw milk of varying purity and also in laboratory-pasteurized milk. It is now possible to suggest that they were probably the major component in the alkali-forming and inert groups which have been shown by Ayers & Johnson (6) and others to constitute a notable proportion of the bacterial flora of milk. Of our isolates, over 90% of the alkali formers were *Alcaligenes* in the case of both raw and pasteurized milk.

The strains isolated in the present work were of two types which will be designated *Alcaligenes viscosus* and *A. tolerans* n.sp. The first was detected among the predominant bacteria in eleven of the thirteen samples of certified and in eight of the ten samples of bulked raw milk examined, but never in laboratory-pasteurized milk. *A. tolerans* was found less frequently in raw milk, but was detected in both certified and bulked milk after pasteurization in the laboratory. It multiplied rapidly in various samples after pasteurization.

Alcaligenes viscosus

This name, published by Weldin (7), is the most recent of those that have been given to an organism which is known chiefly as a causative agent of ropiness in milk and cream. There has been some disagreement concerning its characteristics. A culture of a sugar-fermenting organism named *Bacillus lactis viscosus* Adametz, and assumed to be *Bacterium lactis viscosum* Lehmann & Neumann (8), was for a considerable period distributed by the Kral collection (Magnusson (9)). These names are listed among the synonyms of *Alcaligenes viscosus* in *Bergey's Manual* (10), as is also *Bacterium visco-coccoidium*, which was proposed by Buchanan & Hammer (11) for a sugar-fermenting organism which produced slime in

milk. However, the majority of workers adopt the attitude, shared by us, that sugar fermenters should be excluded from the species *Alcaligenes viscosus*, since a homogeneous group is thereby obtained. A good description of the species is given by Long & Hammer (12), but, on the basis of the present work, it is possible to clarify their characterization and to extend it in certain directions.

Characterization

Lack of motility is a differential character of some importance. Adametz (13), who is generally credited with the original characterization of this type of organism, found it to be slightly motile, but all observers in the last fifty years appear to have failed to detect motility. This indicates that Adametz is now presumed to have made an error, although *Bergey's Manual* (10) still refers to his report of motility

Every culture tested on fat agar in the present work showed lipolytic activity. This confirms Long & Hammer's (12) report that the ability to bring about fat hydrolysis is one of the salient properties of this species.

Long & Hammer describe a transient and inconsistent acid formation from sugars in bouillon; none of our strains produced acid from glucose or lactose in peptone water. The acidification of a glucose ammonium salt medium, mentioned by Long & Hammer, could be explained by the assimilation of ammonia and the production of CO_2 during growth.

Nitrate, according to Long & Hammer, is not reduced; a proportion of our strains reduce it to nitrite.

The majority of the cultures examined in the present work did not produce a ropy condition in milk or other media at favourable temperatures (10–22°). To separate such strains from the others as a distinct variety, *dissimilis*, as proposed by Long & Hammer, is quite impracticable. The viscosity of cultures is an indefinite property; it varies in degree from strain to strain and among colony selections from a single culture. Capsule formation has been described by several writers, but definite capsules were rarely detected either in ropy or non-ropy cultures of our series. The mucilage, which seems to be formed more or less abundantly on any medium that permits growth, is normally amorphous.

Citrate utilization

The production of alkalinity in milk is commonly regarded as an important diagnostic character of this and other members of *Alcaligenes*. Nevertheless, some of the strains that we identify with *A. viscosus* change the pH of milk only slowly and erratically. Since the alkaline reaction in milk is attributed (Ayers, Rupp & Johnson (14)) to the oxidation of citrate, the constancy of citrate utilization was examined. For this purpose a useful medium was found to be Simmons's (15) citrate-salts agar with a reduced concentration of brom-thymol blue.

When plated on that medium some strains developed relatively easily and produced alkalinity by oxidation of the citrate. The colonies appeared more slowly and often in smaller numbers than they did on peptone-meat extract agar, but there was little difference in the final size of the colony on the two media. The longer lag on citrate agar was evident even when the inoculum was taken from a citrate culture, thus indicating that lack of adaptation to citrate was not the factor responsible. Strains of the type just described produced alkalinity in milk. They gave abundant growth and an alkaline

reaction in Koser's(16) citrate inorganic salts solution, although in that medium small inocula might fail to develop.

Other strains were unstable in citrate utilization. When plated on citrate agar they formed minute colonies which did not change the pH. After an interval strongly growing, citrate-oxidizing (alkali-forming) colonies would appear. In some cases only a small proportion of the colonies showed this development, and did so only after plates had been incubated for several days. Organic impurities of agar evidently provided for the weak initial growth because similar colonies developed if citrate was omitted from the medium. The unstable strains frequently failed to grow in Koser's solution, and they were slow and irregular in producing an alkaline reaction in litmus milk. This indicates that the agar provides better conditions for the emergence of a citrate-utilizing variant.

In the case of two of the unstable strains R5 and R50, the variants were further examined. Large citrate-oxidizing colonies on citrate agar were disintegrated in sterile water and streaked on plates or slopes of the same medium. The process was repeated several times, each transfer being made from an isolated colony. Treated thus, the citrate oxidizer appeared to be maintained in a state of purity. Nevertheless, a culture derived from R50 by a series of seven colony selections proved to be still unstable, even if it were kept on citrate agar. The culture, after it had been at room temperature for 6 weeks, gave on plating only a small number of colonies, of which about half failed to oxidize citrate. Several of the small colonies were transferred, and their inability to attack citrate was confirmed. Presumably, in the aged culture citrate had been exhausted and the nutritive conditions had become selective for the citrate-negative variant. A culture derived from R5 by a similar series of seven colony selections appeared to have been stabilized. Several transfers were made on peptone broth and agar, and streaked at various ages on citrate agar, but the citrate-negative type did not appear, at least in detectable proportions.

Starting from the small type of colony which fails to produce an alkaline reaction on citrate agar, single-colony transfers were made in series, using in this case peptone meat extract agar. Stability was tested by heavy inoculation of citrate-agar slopes. The progeny of the two original strains differed in stability. The line derived from R5 invariably threw up the citrate oxidizer; in the thin primary growth there always appeared within 3 weeks one or more of the large alkali-forming colonies. The line derived from R50 was much more stable; only occasionally was a single citrate-oxidizing colony produced.

The variation just described is of the type commonly regarded as mutation. In the strains R5 and R50 the mutation rate for citrate is relatively high in both directions. The two strains differed somewhat in that R50 produced the inert variant with greater frequency and in a more stable condition than did R5; in the production of the citrate-oxidizing mutant they showed the opposite tendency. This study was not pursued in detail, but it reached the point of showing that in *A. viscosus* the oxidation of citrate, and hence the production of alkalinity in litmus milk, is a variable character which in some strains is dependent on the occurrence of a mutation.

The bearing of these findings on the characterization of the genus *Alcaligenes* is outside the scope of the present paper. It may, however, be noted that *A. viscosus* is capable of oxidizing various organic acids, and a preliminary survey has indicated that lactate utilization may be a constant character.

The coccal morphology

A possible source of difficulty in identifying *A. viscosus* is the tendency in certain strains for the cell form to be predominantly coccal. Under the microscope such strains are not unlike a *Micrococcus* or an unusually large *Streptococcus*, more especially as the reaction to Gram's stain may be indefinite. The large diplococci which are at times seen in stained films of raw milk are quite possibly this organism. There are indications in the literature that strains of *Alcaligenes viscosus* have occasionally been identified as micrococci. The descriptions of, for example, *Micrococcus* (*Carphococcus*) *pituitoparus* by Hohl(17) and O'Droma & Grimes(18), *Diplococcus viscosus* by Sato(19) and *Micrococcus lipolyticus* by Stark & Scheib(20) are strongly suggestive of *Alcaligenes viscosus*. In these descriptions reference is made to Gram-negative or ovoid cells, viscous growths, lipolysis, no fermentation of sugars and no liquefaction of gelatin. Among our(2) numerous isolations of micrococci from milk not a single strain showed that combination of culture characters.

Alcaligenes tolerans

This organism, which is of interest on account of its considerable resistance to heat and its occurrence in pasteurized milk, is similar in general character to *A. viscosus*. From that species it is distinguished as follows.

(1) Growth on agar media containing peptone and either meat extract or yeast extract is usually poor and frequently fails completely.

(2) Growth is less easily initiated with citrate or lactate as the only source of carbon.

(3) Natural fats are not hydrolysed, and only a proportion of strains attack tributyrin.

(4) The organism survives heating in milk for 30 min. at 63°. (The survival rate is difficult to measure owing to the erratic growth on agar, especially when heated suspensions are plated.)

Apart from some further differences relating to the suitability of miscellaneous media, no other distinctions between the two types have been detected. Since there are several differential characters, and since in work with dairy bacteria it is useful to distinguish these organisms, they may advantageously be regarded as different species. No description of the heat-resistant form has been found, and for it the name *A. tolerans* is now proposed.

A search for a suitable medium

The study of this organism was hampered by its unsatisfactory growth on agar. Commonly, on agar media prepared from peptone and either meat extract or yeast extract a large inoculum would show a long lag and give rise to only a few colonies which would stop growing when they were still small. Rarely, a vigorous culture not unlike one of *A. viscosus* was obtained on media prepared from the same ingredients and as nearly as possible by the same method. Poor growth has been noted on media prepared from the peptones of various manufacturers and from agars of different origin, including Japanese and New Zealand. Blood agar and bile-salt agar supported little growth. Lactate or citrate with inorganic salts, either as a solution or an agar, gave only a delayed growth and only if heavily inoculated. The addition to such synthetic media of asparagine or glutamic acid or creatine failed to provide for satisfactory growth. Attention was therefore concentrated on peptone meat extract agar in looking for a suitable medium.

An attempt was first made to find an explanation of the batch to batch variation in the

growth-promoting quality of nutrient agar. Differences in pH did not appear to be concerned. Filtration tended to be harmful, but its omission did not ensure a good medium. When the heat treatment in sterilization was increased over the minimum, with a consequence that more precipitation occurred, the medium was improved, but further heating produced deterioration. Broth and agar were prepared separately in double strength with several modifications; when they were combined, some batches of each gave unsuitable media. Meat extract supplied more nutriment than peptone, but it was more liable to be inhibitive. These methods of approach did not yield results except in showing that relatively small variations in the method of preparing the medium might greatly affect the growth of this organism.

The influence of other organisms and bacterial metabolic products was tested. On nutrient-agar plates a satellite growth was sometimes obtained around colonies of other bacteria, but this only happened erratically. Broth cultures of several organisms, including *A. tolerans*, when sterilized by moderate heating and added in small quantities to nutrient agar, slightly improved certain batches of the medium but failed to correct others.

Peptone meat extract agar was examined for nutrient deficiencies and inhibitory constituents. The addition of milk (1–10%) increased the bulk of the final growth. Lactate and citrate, which are obviously energy sources available to the organism, gave the same effect at low concentrations. None of these additions definitely increased the proportion of cells which initiated growth. No benefit resulted from the addition of glucose (sterilized in the medium or filtered), glutamate, arginine, creatine, urea or thioglycollate. The addition of oleic acid, starch or charcoal had no definite effect, thus eliminating an inhibition by fatty acids. Peroxides could not be responsible for the poor growth, since the organism produces catalase actively, and neither fresh blood nor manganese dioxide improved the medium. Treatment of the broth at pH 5 with charcoal failed to improve it, and ether extraction of the agar, peptone and meat extract had no effect. The addition to one of the better batches of nutrient agar of as little as 10% of an unsuitable batch of broth resulted in the complete suppression of growth. This observation strongly suggests the effect of a harmful constituent, not of the absence of something essential.

In looking for an inorganic deficiency in the medium a remarkable growth response was obtained by the addition of 0·01–0·02% ferric citrate or 0·004–0·008% $FeSO_4$, $7H_2O$. Their addition gave the maximum effect if made after sterilization, most obviously in the case of the sulphate. Ferric chloride gave the same effect, and metallic iron stimulated growth in immediately adjacent agar.

The function of iron in the high concentrations found to be optimal is obscure. The organism appears to be well adapted to growth in milk in which the concentration of iron is low (21); in citrate inorganic salts media to which no iron has been added, a good crop of cells may be produced if growth can be initiated by means of heavy inoculation. That iron acts as a carrier of another essential metal is unlikely because it did not give complete correction of all batches of agar, and because various trace elements (Mn, Cu, Zn, B, Mo) were found to be ineffectual. On the best batches of nutrient agar soluble iron had no obvious influence.

The work as a whole indicated that the factor responsible for the variation in agar media is some inhibitory substance rather than a nutrient deficiency. Whether a toxic factor

occurs already in the sterile medium or is formed only during the growth of the organism is not clear, and no evidence was secured on the role of iron in eliminating the suggested inhibition.

The best agar medium evolved in this work contains 0·5% peptone, 0·3–0·5% meat extract, 0·3% ammonium lactate (from a 60% syrup), with the addition, preferably after sterilization, of 0·02% iron citrate, the final pH being 7. The selection of a suitable bottle of peptone is an advantage. This medium is not invariably satisfactory for quantitative uses, but it appears to be sufficiently reliable for the isolation and propagation of the organism.

Since this work was done Stocker(22) has reported similar observations on another bacterium. A strain of *Shigella flexneri* failed to grow on serum agar or on some batches of nutrient agar. The inhibition could be entirely reversed by ferrous or ferric salts, but unlike the case of *Alcaligenes tolerans* described above, a substance formed by the organism itself, and also by other bacterial species, was found to have the same effect as iron.

Characterization of Alcaligenes tolerans *n.sp.*

(Name derived from ability to tolerate high temperatures.)

Cells rod-shaped to coccal, in dry films usually 0·7–1 μ thick, at times up to 2 μ, often in pairs and short chains, non-motile, Gram-negative or -variable, may contain bodies unstainable with fuchsin.

Agar (peptone-meat extract). Growth often poor or absent; improved by adding iron salts (e.g. 0·02% ferric citrate) and organic acids (e.g. 0·3% ammonium lactate). Colony round, raised, glossy, viscid to friable, unpigmented or, on ageing, faintly brown.

Gelatin. No liquefaction.

Broth. Growth occurs at the surface; produces turbidity and sediment.

Milk. Alkaline or, less commonly, unchanged in reaction; may be weakly ropy.

Sugars are not fermented. Diastase is not produced. Lactate and citrate are usually oxidized, but not readily with NH_3 as the only nitrogen source. There is no visible action on casein (in agar). Natural fats are not hydrolysed; a proportion of strains hydrolyse tributyrin. Nitrate is reduced to nitrite by some strains and not by others; no reduction to NH_3 or N_2. No formation from peptone of indole or H_2S has been detected. Catalase is produced.

Strictly aerobic in glucose agar.

Growth occurs at 10 and 37°; optimum about 30°; growth at 40° variable.

Survive in milk for 30 min. at 63°.

Isolated from dairy equipment and milk, especially pasteurized milk.

SUMMARY

Using non-selective methods of isolation, the species that was detected with greatest frequency in raw milks of varying purity is *Alcaligenes viscosus*. The majority of the cultures do not produce a ropy condition in milk or other media. In some strains the oxidation of citrate, and hence the production of alkalinity in milk, is slow and erratic, being dependent on the occurrence of a mutation. The only Gram-negative rod detected in laboratory-pasteurized milk was *A. tolerans* n.sp., a description of which is given. That species is similar in general character to *A. viscosus* but differs principally in its greater

resistance to heat, its inability to attack fats, and its erratic and usually poor growth on laboratory media. Greatly improved growth on nutrient agar is obtained by the addition of lactate and relatively high concentrations of ferrous or ferric salts.

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637.16

485. THE EFFECT OF TEMPERATURE ON THE DEVELOPMENT OF BROKEN CREAM

By M. JEAN STONE

National Institute for Research in Dairying, University of Reading

(With 3 Figures)

INTRODUCTION

In an earlier publication (1) it was stated that *Bacillus cereus* var. *mycoides*, the aerobic spore-forming organisms causing 'broken' or 'bitty' cream, were present in 75% of raw farm milk samples examined and in all the commercially pasteurized and bottled samples. Although our observations were limited to sixty samples of raw milk from fifteen different farms and to fourteen samples of pasteurized milk, it is becoming increasingly evident from our own experience and the comments of other workers that to exclude spores from milk is virtually impossible in practice. Thomas, Jones-Evans, Jones & Thomas (2) found that the proportion of aerobic spore-formers to other micro-organisms was greatest on utensils sterilized by steam, and from the work which has been published on the destruction of spores on dairy utensils by chemical means it seems that in practice this method may also fail.

At present the most practicable means of avoiding milk spoilage by *B. cereus* and *B. mycoides* seems to be efficient cold storage of the milk before distribution to the consumer. Some preliminary experiments described previously (1) showed that if milk containing spores of *B. cereus* were stored at 5° C. or lower for 24 hr. after pasteurization, broken cream did not develop within a further 24 hr. at a temperature as high as 18° C. In these experiments an initial inoculum of approximately 10,000 spores per ml. of 'sterile' milk was used. The work described here is a continuation of that begun in 1950 on the rate of growth of *B. cereus* in milk and the rate of development of broken cream at different temperatures, using levels of inoculum of the order of 1–50 spores per ml. which it was considered were comparable to those found in practice. In addition, samples of bottled pasteurized milk from two local creameries were examined under similar conditions of storage. One creamery (Creamery A) had received complaints of broken cream during spells of hot weather while the other (Creamery B) had not, but a few preliminary tests showed that milk from both creameries contained spores of *B. cereus*. A comparison was made therefore between the rate of growth of *B. cereus* and the development of broken cream in 'sterile' milk inoculated with spores of *B. cereus* and the development of the fault in commercially pasteurized milk from these two sources. From our results we hoped that any factors, other than temperature, which affected the growth of *B. cereus* in milk from one source but not from another would come to light.

EXPERIMENTAL

Preparation of spore suspensions

For the work with pure cultures, over fairly long periods, a stable suspension of spores of the required strain of *B. cereus* was used. The suspension was prepared by growing the

organism on the surface of 3% nutrient agar in a Roux bottle at 30° C. for 1 week, when sporulation was well advanced. The growth was washed off with 50 ml. sterile distilled water into a sterile 150 ml. conical flask containing glass beads and shaken by hand for 5–10 min. to break up clumps. The suspension was centrifuged for 10 min. at 2500 r.p.m. to remove the large particles. Finally, the supernatant liquid was transferred to a screw-capped bottle in which it was heated to 80° C. for 10 min. and subsequently stored in the refrigerator.

Suspensions of *B. cereus* prepared by this method contained about 2,000,000 spores per ml., and the count remained unchanged for several months.

The rate of growth of pure cultures of Bacillus cereus *and development of broken cream in 'sterile' milk*

Milk for use in this work was sterilized as previously described (1). A sample of the milk from a single cow was transferred from the bucket immediately after milking to a sterile narrow-necked heat-resistant glass bottle. The milk was pasteurized at 63° C. for 30 min., cooled and held overnight at 18° C. Next morning it was repasteurized and then stored in the refrigerator until required for use.

The effect of storage temperature. The required number of spores, obtained by dilution of the stock suspension, was added to 200 ml. of sterile whole milk in the narrow-necked bottle in which the sample had originally been taken. The milk was preheated in a water-bath to 65° C. using a similar bottle containing water as a control. The cotton-wool plug was replaced by a rubber stopper and the bottle immersed in a constant-temperature water-bath at 62·5° C. for 30 min. The milk was then cooled in iced water for 15 min., and the 200 ml. sample was then divided into three subsamples and stored in 8 × 1 in. heat-resistant tubes at 5, 10 and 15° C. for 24 hr. After storage, each subsample was again distributed into three tubes which were incubated for a further 24 hr. at 15, 18 and 22° C. and observed for broken cream and sweet curdling.

Colony counts were estimated immediately after pasteurization and again after 15 min., 6 hr., 24 hr., 30 hr., and 48 hr., from triplicate poured plates. Counts of spores were made at the same time by heating 5 ml. quantities of the milk to 80° C. for 10 min. before plating. Throughout this work 3% nutrient agar was used to limit the spreading growth of the organisms (3,4).

The effect of pasteurization. Curran & Evans (5) have noted the accelerating effect of sublethal heating upon the germination of bacterial spores, a phenomenon quite distinct from heat activation of spores (6,7,8). Preliminary work with the two strains of *B. cereus* (B.C. 5 and B.C. 60) used showed that spores which had been heated to pasteurization temperature in milk, germinated faster than those which had received no heat treatment. Within 2 hr. of pasteurization 96·35% of the spores of B.C. 5 and 99·99% of the spores of B.C. 60 had germinated at 18° C., while without heat treatment only 48 and 84·3% germination occurred in 2 hr. at the same temperature. In order to ascertain whether the delay in germination would appreciably affect the development of broken cream, each experiment was repeated omitting the pasteurization process.

The effect of size of inoculum. Four levels of inoculum were used, at 50, 25, 10 and 1 spore per ml. Inocula of the order of 1 spore in 10 or 100 ml. were not included, because of the obvious difficulties of estimating numbers and the large quantities of milk required to avoid diluting out the organism from any one portion.

The rate of growth of Bacillus cereus *and development of broken cream in bottled pasteurized milk*

Pint bottles of pasteurized milk taken from the filler were used. Triplicate 50 ml. subsamples were transferred with a pipette into 8 × 1 in. heat-resistant tubes closed by rubber stoppers. Two of the tubes were taken to the laboratory in an insulated box containing ice, where one was stored for 24 hr. in a constant temperature water-bath at 5° C. and the other at 15° C. The third tube was kept near to the thermometer in the creamery cold store for 24 hr. before being taken to the laboratory. The three subsamples were further subdivided into three 6 × ¾ in. tubes, one of which was incubated at 15° C., one at 18° C. and one at 22° C. for another 24 hr. and observations made for broken cream. The initial colony count of one of the subsamples brought direct to the laboratory was determined on yeast dextrose agar. The count of *B. cereus* was determined from these plates on colonial appearance. With experience the characteristic ground-glass colonies were readily distinguishable. The method of Miles & Misra (9) was used for estimating the total colony count and numbers of *B. cereus* present after 24 and 48 hr. This method is economical in time and materials with large numbers of samples, and also facilitates the counting of *B. cereus* whose colonies are more easily distinguished on the surface than in the depths of an agar medium. The plates were allowed to dry before use as suggested by Davis (10) to avoid the development of spreading colonies.

From each bottle of pasteurized milk a 25 ml. portion was also transferred by pipette to an 8 × 1 in. heat-resistant glass tube. This was heated to 80° C. for 10 min. at the creamery, using a similar tube containing water as control. On arrival at the laboratory this sample was incubated at 22° C. and observed for the development of broken cream and sweet curdling. After 48 hr. one loopful of the milk was transferred to 9 ml. of ¼ strength Ringer's solution and one loopful of the suspension streaked on egg-yolk agar (11). The plates were incubated at 30° C. for 24 hr. and examined for the presence of the characteristic zones produced by *B. cereus* and *B. mycoides*. These heated portions were used as controls to check negative results which might be obtained from the unheated samples.

RESULTS

The rate of growth of Bacillus cereus *and the development of broken cream in milk*

The effect of storage temperature. Nine different combinations of temperature were used in these experiments in an attempt to reproduce the best and worst conditions under which milk might be kept up to the time of delivery and during the first 24 hr. in the hands of the consumer, at all seasons of the year. Observations were discontinued after 48 hr., as this was considered to be the maximum length of time during which milk would be kept before consumption. The work was repeated with three strains of *B. cereus*; B.C. 5 was isolated from farm-pasteurized milk, B.C. 60 from raw farm milk and 484 from commercially pasteurized and bottled milk. The last culture was a strain of *B. cereus* var. *albolactis*, kindly supplied by Miss Bird of the N.A.A.S., Bristol. Each culture was tested at the four levels of inoculum, making a total of twelve experiments.

No marked differences were observed between the results of ten of these experiments and they are considered together. The results confirmed our earlier observation (1). It was found that when milk containing spores of *B. cereus* was held at 5° C. for 24 hr. following pasteurization, the subsequent critical temperature above which broken cream

developed within a further 24 hr. was between 18 and 22° C. After storage at 10° C. the critical temperature was between 15 and 18° C., and after storage at 15° C. the critical temperature was below 15° C. The rates of growth of the organisms throughout the 48 hr. observation period have been plotted and an example of the curves obtained is shown in Fig. 1, together with observations on the development of broken cream at the nine storage temperatures.

The effect of pasteurization. Four levels of inoculum were again used for each of the three cultures, but pasteurization was omitted. The results obtained indicated that heat treatment does in fact accelerate the development of broken cream. Out of a total of twelve experiments it was found that in five cases the samples did not develop broken cream as rapidly as is indicated in Fig 1. The fault did not appear within 48 hr. in milk containing 10 spores or 1 spore per ml. of B.C. 60, except in the portions incubated at 22° C.

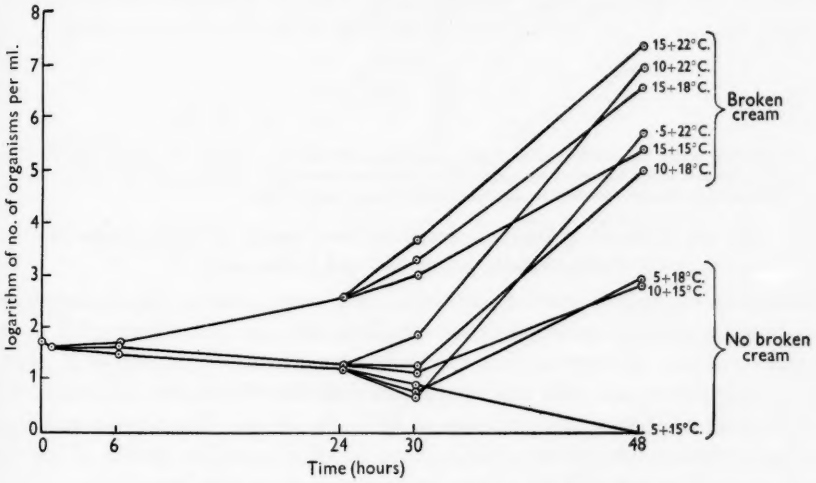

Fig. 1. Development of *B. cereus* (B.C. 60) in sterile whole milk.

following preliminary storage at 5, 10 and 15° C. Where the inoculum was of the order of 25–50 spores per ml. no delay in the rate of growth or production of the fault was observed. No differences were noted in the rate of development of broken cream between pasteurized and unheated spores of 484.

With B.C. 5, which appeared to be less active than the other two strains of *B. cereus* used, the delay was even more pronounced, for only when 50 spores per ml. were present initially was the rate of growth comparable with that shown in Fig. 1. When the initial number of spores was 25 or less per ml., broken cream failed to develop within 48 hr., whatever the temperature of storage.

The effect of the size of inoculum. It was found that some decrease in the rate of development of broken cream occurred when the initial numbers of the spore-former were reduced. Again, this was particularly noticeable with B.C. 5. In two cases, milk which was inoculated with spores of B.C. 5 and pasteurized at 63° C. for 30 min. did not develop the fault within 48 hr. at any temperature. This occurred with an initial inoculum of 10 spores or 1 spore per ml. When there were 25 spores per ml. present initially, the production of the fault was not delayed. On the other hand, no appreciable differences

occurred in the rate of growth of B.C. 60 and 484 after pasteurization, even when the inoculum was of the order of 1 spore per ml.

The results of the twenty-four experiments in which three individual strains of *B. cereus* were tested at four levels of inoculum, both after pasteurization and with no heat treatment, are summarized in Table 1.

Table 1. *The development of broken cream in pasteurized and unpasteurized milk after* 48 *hr. storage*

Strain of *B. cereus* used	Size of inoculum (spores per ml.)	Pasteurized 63° C./30 min.	Not pasteurized
B.C. 5	50	+	+
	25	+	−
	10	−	−
	1	−	−
B.C. 60	50	+	+
	25	+	+
	10	+	±
	1	+	±
484	50	+	+
	25	+	+
	10	+	+
	1	+	+

+ = Broken cream observed in all portions except those stored at 5 + 15° C., 5 + 18° C., 10 + 15° C.
± = No broken cream observed except in those portions stored at 5 + 22° C., 10 + 22° C., 15 + 22° C.
− = No broken cream observed in samples stored at any temperature.

The rate of growth of Bacillus cereus *and development of broken cream in commercially pasteurized and bottled milk*

Twenty-three samples of bottled pasteurized milk were examined; fifteen samples were taken from Creamery A on five different occasions and nine from Creamery B on three different occasions. In every case it was found that the rate of growth of *B. cereus* in commercially pasteurized milk was comparable with that of the pure cultures in sterile milk following laboratory pasteurization at 63° C. for 30 min. The number and type of the other organisms present appeared to have no effect upon the growth of the spore-former. It is of interest that the colony counts obtained on milk from Creamery B were consistently higher than those from Creamery A.

Figs. 2 and 3 illustrate the growth curves of the whole of the bacterial flora and those of *B. cereus* alone obtained from one sample from each source. It was found that the average temperature of the creamery cold stores was approximately 10° C., and this temperature has been used in labelling the charts. The appearance of the curves from each of the twenty-three samples is almost identical and therefore only two are shown.

It may be noted here that the number of *B. cereus* colonies counted on the initial poured plates was rarely less than 10 per ml., and counts as high as 100 per ml. were sometimes obtained. These values refute the generally accepted statements (12,13) that milk immediately after production or pasteurization contains very few spores.

As would be expected, broken cream developed and colonies of *B. cereus* were isolated from all the samples heated to 80° C. for 30 min. and incubated directly at 22° C.

DISCUSSION

The results presented in this paper show that once milk has become infected with spores of *B. cereus* the rate at which these organisms multiply and give rise to the fault known as broken cream depends almost entirely upon the temperature of storage. Thus, in

fifteen out of twenty-four cases, it was shown that when sterile milk inoculated with spores of *B. cereus* is stored at 5° C. for 24 hr., broken cream develops within a further 24 hr. when the temperature is raised to 22° C. but not at 15 or 18° C. With a preliminary

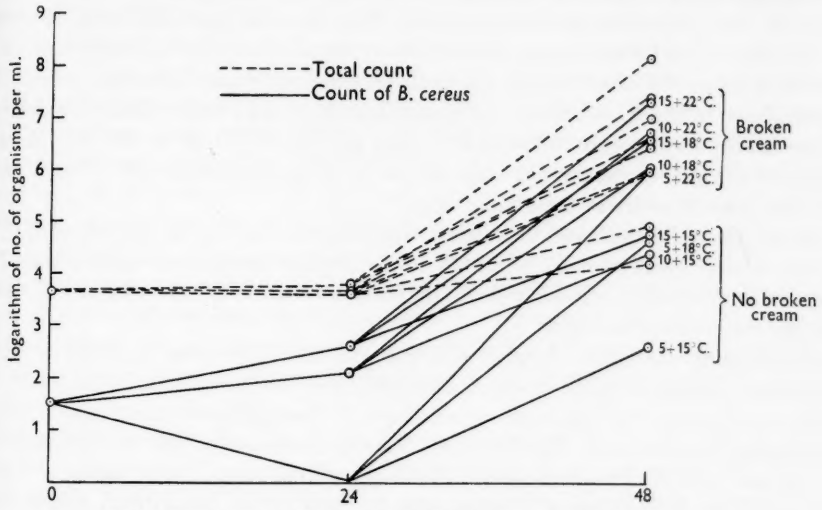

Fig. 2. Development of *B. cereus* in bottled pasteurized milk (Creamery A).

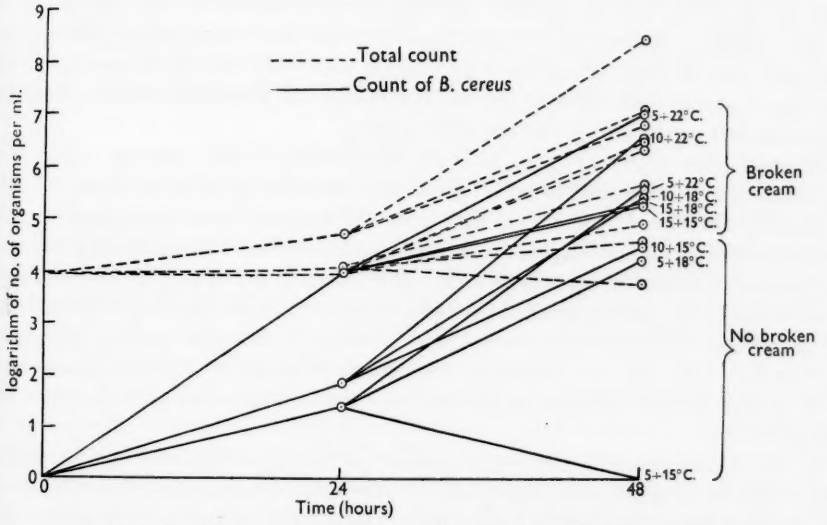

Fig. 3. Development of *B. cereus* in bottled pasteurized milk (Creamery B).

storage at 10° C. the fault developed within a further 24 hr. at 18 or 22° C., but not at 15° C., and with preliminary storage at 15° C. the fault appeared within 24 hr. at all three subsequent incubation temperatures.

Our investigations of the factors affecting the occurrence of broken cream also included observations on the effect of the number of spores present initially, the variation between

strains of the organism used and the effect of heat treatment. The results indicate that all of these factors singly or combined may affect the rate at which broken cream develops. The most important single factor seems to be that of heat treatment, for in five of the nine experiments a marked delay in the production of broken cream occurred, because the spores had undergone no heat treatment. Thus, in milk inoculated with 25 spores per ml. of B.C. 5 no broken cream occurred at any temperature when heat treatment was omitted, whereas the rate at which the fault developed after pasteurization was similar to that shown in Fig. 1. Similarly, the appearance of the fault was considerably delayed in four instances where only 10 spores or 1 spore per ml. of B.C. 60 or 484 were present initially, while milk containing the same number of spores and heated to 63° C. for 30 min. developed broken cream at the normal rate.

The remaining four samples, inoculated with 10 spores and 1 spore per ml. of B.C. 5, only two of which were pasteurized, did not develop broken cream at any temperature. The difference in this case was probably due to the less active strain of *B. cereus* used, combined with the low levels of inoculum.

By comparing the results obtained with pure cultures of *B. cereus* in sterile milk and those from samples of commercially pasteurized and bottled milk it is clear that, in practice, methods of handling milk are conducive to the rapid multiplication of *B. cereus* at favourable temperatures. In every one of the fifteen samples of milk from Creamery A and the nine samples from Creamery B, broken cream developed in all portions except those stored at 5° C. followed by incubation at 15 or 18° C., or at 10° C. followed by 15° C. It is obvious that, in bulk milk from widely different sources, the individual strain of *B. cereus* has no influence upon the development of broken cream. In our limited experience the number of spores present in milk immediately after pasteurization is greater than has been suggested by previous workers (13,14), so that in commercial milk supplies it seems that sufficient spores are present to germinate rapidly after heat treatment and produce their characteristic spoilage.

Obviously, the degree of spore infection of raw-milk supplies and the effect of the pasteurization process upon the rate of germination of those spores are beyond the control of the milk distributor. Indeed, the only controllable factor in the prevention of broken cream seems to be that of temperature, and the results presented here confirm our previous statement (1) that if milk containing spores of *B. cereus* is stored at 5° C. or lower before distribution, it may subsequently be stored at a temperature as high as 18° C. for at least 24 hr. without broken cream developing. However, it has also become increasingly evident that, when the temperature of milk rises to 22° C. or more, as it is almost bound to do in hot weather in most households, the prevention of the fault does not seem possible.

In our opinion the milk distributor should not rely upon the long keeping qualities which may be obtained with milk produced and handled under present-day conditions, but should make every effort to handle milk quickly with the best possible refrigeration.

It is of interest that broken cream occurred as readily in milk from Creamery B as in milk from Creamery A. A possible explanation of absence of broken cream in milk from Creamery B may be, as has been suggested by Egdell (14), that, unless the condition is well advanced, the fault may pass unnoticed. However, from our work with milk from the two sources, it was hoped that some fairly conclusive evidence would be obtained that certain conditions (found in milk from Creamery B but not from Creamery A)

directly suppress the growth of the spore-former and hence the appearance of broken cream. Davis(15) suggested that in the presence of other organisms, particularly acid-producers, the growth of the spore former was inhibited. We have therefore given attention in a few preliminary experiments to the associative growth of *B. cereus* and *Streptococcus lactis* in milk.

Three separate strains of *B. cereus* were grown in turn with three strains of *Str. lactis*. Using levels of inoculum of 10,000, 100 and 1 cell per ml., 50 ml. quantities of milk in 8 × 1 in. heat-resistant glass tubes were inoculated with the two organisms in all proportions. After mixing, 10 ml. of the milk was poured into a test-tube and 1 ml. of methylene blue added. The two tubes were then placed in a constant-temperature water-bath at 22° C. and the times to reduction of the methylene blue, precipitation with 68% alcohol and clot-on-boiling were recorded. The formation of broken cream was also noted. The initial colony count of each organism was estimated from poured plates of the appropriate dilution tubes from which the milk was inoculated. At the end-point of the methylene-blue test and again at clot-on-boiling end-point, the numbers of spore-formers and streptococci present were estimated by drop-plate counts(9) on the surface of yeast dextrose agar and the same medium containing 0·01% thallium acetate.

Our results indicate that *Str. lactis* has no inhibitory effect upon the growth of *B. cereus* in milk at 22° C., or upon the development of broken cream. The number of *B. cereus* present at the end-point of methylene-blue and clot-on-boiling in milk containing *Str. lactis* was of the same order as in milk inoculated with a pure culture of *B. cereus* and incubated at 22° C. It appears, therefore, that, where milk becomes infected with spores of *B. cereus*, the only means by which broken cream may be controlled is efficient refrigeration at the processing depot, combined with quick handling and the coolest possible storage in the hands of the consumer.

SUMMARY AND CONCLUSIONS

Three strains of *B. cereus* were grown in milk at nine different temperature combinations. Broken cream could be prevented by holding milk at 5° C. or lower for 24 hr. before transferring to 15 or 18° C. for a further 24 hr., but with a subsequent period of storage at 22° C. the fault was not prevented. Broken cream developed after storage at 10° C. followed by 18 and 22° C., but not in milk kept at 10° C. and then transferred to 15° C. After preliminary storage at 15° C. the fault appeared within a further 24 hr. regardless of the subsequent storage temperature.

With two strains of *B. cereus* it was observed that pasteurization accelerated spore germination and hence the development of the fault. With low levels of inoculum there was delay in the appearance of the fault with one strain of the organism.

In twenty-four samples of commerically pasteurized milk it was shown that the fault could be controlled by refrigeration at 5° C. before distribution except when the subsequent temperature was allowed to rise to 22° C.

Contrary to the suggestion made by Davis(15) preliminary observations indicate that the growth of *B. cereus* and the development of broken cream is not inhibited by the presence of acid-producing organisms such as *Str. lactis*.

The author is indebted to Dr A. T. R. Mattick for invaluable advice and criticism.

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486. THE ACTION OF THE LECITHINASE OF *BACILLUS CEREUS* ON THE GLOBULE MEMBRANE OF MILK FAT

By M. JEAN STONE

National Institute for Research in Dairying, University of Reading

(With 3 Figures)

INTRODUCTION

The mechanism by which *Bacillus cereus* and its variants produce the fault known as broken cream in raw and pasteurized milk has never been completely understood. In an earlier publication (1) it was noted that the production of this type of spoilage by *B. cereus* and the production of the enzyme lecithinase by this organism were related. Since *B. cereus* and its variants are the only aerobic spore-forming bacilli to cause this fault, and as they are also the only two common milk organisms to produce lecithinase, it was suggested that the development of broken cream was caused partly, at least, by hydrolysis of the lecithin of the fat-globule surface membrane (2,3,4). This leads to destabilization of the fat globules and the characteristic appearance of broken cream. Further support for this was provided by three strains of *B. cereus* which did not produce lecithinase and also failed to produce broken cream when added to sterile milk. However, all attempts to reproduce the fault by addition of a concentrated culture filtrate of *B. cereus* to milk or cream were unsuccessful.

A study of the work of Palmer and his colleagues (5,6,7), on the fat-globule membrane, suggested that by using washed cream instead of whole milk or cream, the manner in which broken cream develops might be demonstrated. In washed cream the fat-globule membrane constitutes the only source of lecithin; if it could be shown that the *B. cereus* lecithinase is capable of hydrolysing the lecithin in washed cream it would follow that the development of broken cream involves the breakdown of lecithin at this point.

The method adopted was that used by Perlman (8). While working on methods of fat analysis of mayonnaise he found that a gradual decomposition of lecithin occurred during storage without refrigeration, and suggested that the gradual decrease in lipoid phosphorus was caused by bacterial hydrolysis. He stated that from the hydrolysis of lecithin, free choline, glycerophosphoric acid and fatty acids are formed as end-products. To prove this Perlman made determinations of the presence of free choline in samples of mayonnaise and eggs, both fresh and after storage, by the microtest of Stanek (9) and Supplee (10), which depends on the formation of a crystalline water-insoluble choline poly-iodide.

In the work described here it is shown that the lecithinase of *B. cereus* is in fact capable of breaking down the surface membrane of the fat globule, and evidence is supplied to show that this reaction is at least partly responsible for the development of broken cream.

EXPERIMENTAL

Preparation of washed cream

Twenty ml. quantities of heavy cream (approx. 40% fat) were warmed to about 45° C. in a water-bath, diluted 1:4 with sterile distilled water at the same temperature, and centrifuged at 4500 r.p.m. for ½ hr. The serum layer was removed with a sterile pipette, the remaining cream layer again made up to 100 ml. with sterile distilled water and the suspension recentrifuged. The process was repeated four more times, and after the final centrifuging the cream layer was made up to 50 ml., producing a washed cream containing 15–20% fat. The washed cream was then pasteurized at 63° C. for 30 min. in the tubes in which it was centrifuged, and stored in the refrigerator until required.

Concentration of Bacillus cereus *lecithinase*

One litre of nutrient broth containing 0·2% dextrose was inoculated with 1 ml. of an 18 hr. broth culture of the organism and incubated at 30° C. for 24 hr. It was Seitz filtered to remove the cells and the culture filtrate was concentrated by the method described by Chu (11). The concentration was finally dialysed against glycerol at 0° C. From 1 l. of broth filtrate approximately 50 ml. of concentrated glycerol solution was obtained. This was stored in the ice-chest and retained its activity over a period of 2 years.

The action of Bacillus cereus *lecithinase on the fat globule membrane*

The washed cream was dispensed in 25 ml. quantities in 150 ml. conical flasks. Each flask was inoculated with a double loopful of a 24 hr. nutrient broth culture of one of the following strains of *B. cereus*: B.C. 5, B.C. 60 and a non-lecithinase producing strain, B.C. 93. To two other flasks was added 1 ml. of each of the concentrated filtrates prepared from cultures of B.C. 5 and B.C. 93. The flasks inoculated with *B. cereus*, together with an uninoculated control, were incubated at 22° C. and those with the concentrate added, also with a control flask, were incubated at 37° C. The contents of the flasks were examined daily for 1 week for visual changes in the washed cream simulating the appearance of broken cream in whole milk.

After 1 week's incubation, the contents of each flask were examined microscopically by the method of Cole & Smith (12), who used alkaline Nile blue sulphate for the examination of fat globules. Extracts of the washed cream from each flask were made by a modification of the technique used by Perlman (8). The free fat was first removed by filtering the cream through an ordinary paper filter. The filtrate was evaporated almost to dryness, extracted with 15 ml. absolute alcohol, evaporated over a water-bath and the residue dissolved in 10 ml. distilled water. If the solution appeared cloudy it was filtered once more. A drop of the aqueous filtrate was placed on a glass slide next to, but not touching, a drop of potassium iodide reagent (8,9,10), covered by a glass slip and examined microscopically for the presence of crystals of choline poly-iodide at the junction of the two liquids.

RESULTS

After 3 days' incubation it was observed that the washed cream inoculated with B.C. 5 and B.C. 60 had assumed a distinctly 'bitty' appearance with large particles of the cream floating in a clear watery serum. The particles at this stage had a white 'curdy' appearance

not unlike broken cream in whole milk. At the end of a week the effect was even more pronounced. The cream appeared exactly like freshly churned butter with large 'grains' floating in a watery serum. The change in the appearance of washed cream containing the concentrated lecithinase of B.C. 5 was not so marked. After 3 days' incubation the cream appeared quite bitty, but no further change occurred during a further 4 days' incubation. No visual change occurred in the control flasks or in the washed cream containing either the concentrated filtrate of B.C. 93 (the non-lecithinase-producing strain) or the organism itself.

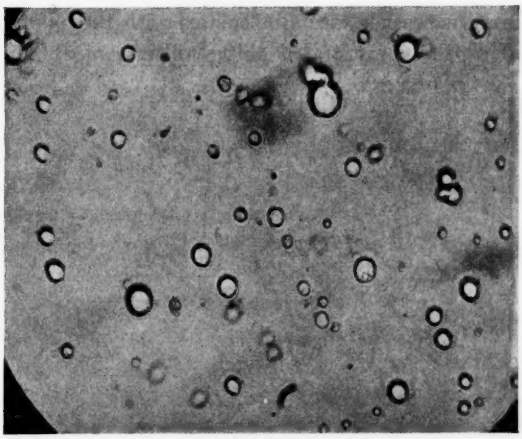

Fig. 1

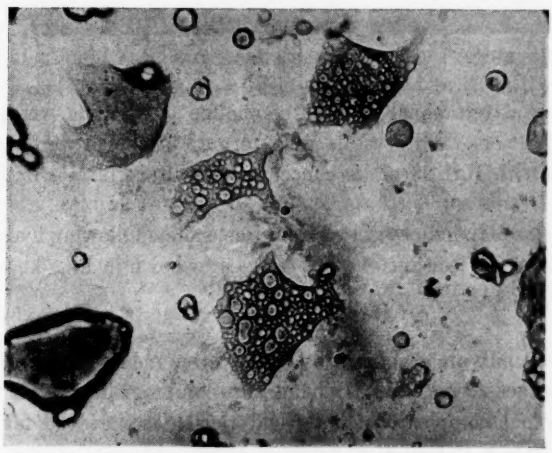

Fig. 2

Microscopic examination of washed cream

The microscopic preparations from cream inoculated with B.C. 5 and B.C. 60 and those from cream to which the concentrated filtrate of B.C. 5 had been added, showed that most of the fat-globule membranes had been removed. The fat (which was stained pink) had aggregated into large masses in which droplets of the serum were incorporated,

similar to phase-inversion in butter churning but on a smaller scale. The fat globules in the washed cream containing B.C. 93 or its concentrated filtrate and in the uninoculated cream were quite normal in appearance, being perfectly spherical with a distinct surface 'membrane'.

The normal fat globules are seen in Fig. 1, and those showing the effect of the lecithinase in Fig. 2.

Qualitative estimation of lecithin hydrolysis in washed cream

The results of the microtest for the estimation of free choline indicated that hydrolysis of lecithin had occurred in washed cream inoculated with B.C. 60 and B.C. 5, and also in the cream to which the concentrated culture filtrate of B.C. 5 had been added.

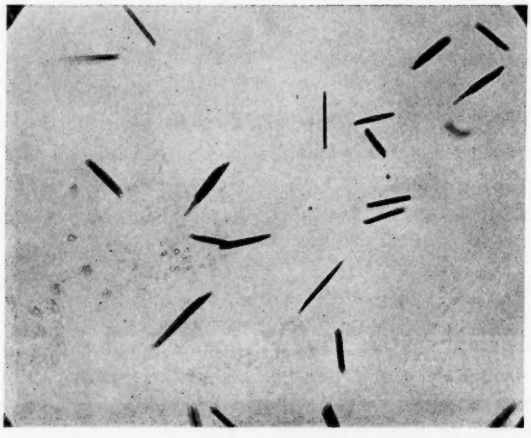

Fig. 3

Characteristic brown, tetragonal crystals of choline poly-iodide were observed in the microscopic preparations. No crystals were formed in the preparations from washed cream inoculated with B.C. 93 or its concentrated culture filtrate or from the washed cream controls.

Crystals of choline poly-iodide formed from free choline following hydrolysis of lecithin in washed cream, by the lecithinase of *B. cereus* are shown in Fig. 3.

DISCUSSION

The fat globules in milk are stabilized by a protective membrane consisting of lecithin, which is very surface-active, and is chemically combined with milk protein forming a casein phospholoid complex which is held tenaciously at the fat-globule surface. According to Rimpila & Palmer (6) all milk plasma constituents are removed from cream after four washings, leaving only the membrane proper adsorbed to the fat globules. This membrane is not removed by as many as twelve washings.

Further evidence is provided here that broken cream develops as a result of the breakdown of the fat-globule membrane by the lecithinase produced by *B. cereus*. By allowing the causative organism to grow in washed cream a condition simulating that of broken cream in whole milk was observed. Furthermore, the microscopic appearance of fat

globules from washed cream in which *B. cereus* had been growing indicated that the fat-globule membranes had been in some way disrupted. At the same time it was shown that hydrolysis of lecithin had occurred in the washed cream inoculated with *B. cereus*. Although the visual effect of a concentrated culture filtrate of *B. cereus* upon washed cream was not so marked as that produced by the organism itself, the microscopic appearance of the fat globules was identical. It was shown also that the lecithin of the fat-globule membrane was hydrolysed by the concentrated preparation of the enzyme.

The fact that *B. cereus* lecithinase breaks down the fat-globule membranes in washed cream does not prove conclusively that this is the mechanism by which broken cream is produced in whole milk. However, the evidence is supported by the fact that a non-lecithinase-producing culture of *B. cereus*, and the concentrated filtrate prepared from it, both failed to produce any change in washed cream and did not bring about any break-down of the fat-globule membranes.

Bearing in mind the type of visual change produced in washed cream from which all milk plasma constituents have been removed, it seems likely that in whole milk the appearance of broken cream is caused by the disruption of the fat-globule membrane combined with some coagulation of protein by a second enzyme produced by *B. cereus*.

SUMMARY AND CONCLUSIONS

An effect simulating broken cream in whole milk was produced when *Bacillus cereus* was grown in washed cream at 22° C. A similar but less marked effect was obtained when a concentrated culture filtrate of *B. cereus* was added to washed cream.

Microscopic examination of washed cream to which *B. cereus* or its concentrated filtrate had been added showed that the fat-globule membranes had been broken down. The hydrolysis of lecithin in washed cream in which *B. cereus* had grown was demonstrated by qualitative estimation of free choline.

Since these results could not be reproduced when a non-lecithinase-producing strain of *B. cereus* or its concentrated culture filtrate was added to washed cream, it appears that the hydrolysis of the lecithin of the fat-globule membrane is at least partly responsible for the formation of broken cream.

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487. STUDIES ON THE ORIGIN OF OXIDIZED FLAVOUR IN WHOLE MILK

By E. G. PONT

Dairy Research Section, C.S.I.R.O., Melbourne, Australia

Cardboard, metallic, oily and tallowy flavours are serious defects in market milk in many countries, including Australia (1). They frequently develop in the order given, and because they are believed to arise from a series of oxidation reactions the collective term 'oxidized flavour' is now commonly applied to them. Since the work of Thurston, Brown & Dustman (2) and Swanson & Sommer (3) it has been widely accepted that spontaneous or copper-induced oxidized flavour in milk arises from oxidation of phospholipin in the fat-globule membrane. This theory has received further support from a belief that milk fat is stable towards the mild oxidizing conditions in milk which favour the defect (4) and from the apparent non-occurrence of oxidized flavour in skim milk.

PART I. THE ROLE OF FAT OXIDATION

Earlier workers attributed oxidized flavour in whole milk to oxidation of the fat, but this theory was discredited largely by the failure of classical chemical methods such as the Kreis test, the iodine number, and iodometric peroxide tests to detect measurable changes in milk fat when oxidized flavour had developed. In addition, there exists a widespread misconception of the degree of oxidation necessary for flavour changes in milk fat. A typical view is expressed by Hunziker (5) who states: 'Milk fat when in contact with oxygen or air passes through an initial period during which oxygen absorption while not entirely absent is very slow and practically imperceptible. This is called the induction period. During this period the fat remains free from organoleptically detectable tallowy flavour.' In fact milk fat, by the time it even approaches the end of the induction period, has an intense tallowy flavour and is inedible. Experience in this laboratory has repeatedly shown flavour changes in the very earliest stages of oxidation. Furthermore, use of the sensitive ferric thiocyanate method of Loftus Hills & Thiel (6) permits measurement by chemical means of the oxidation of the fat from the time it leaves the udder. It has been shown by Pont (7) that fat from milk exposed to daylight begins to show an oily-tallowy flavour at a peroxide value of about 0·3. (Fresh unoxidized fat usually has a peroxide value of about 0·02.) The new thiobarbituric acid reagent for evaluating fat oxidation is also capable, according to Patton & Kurtz (8), of measuring the degree of oxidation in milk fat corresponding to the first indication of flavour change. This test has been applied by Dunkley (9) directly to whole milk and has been found to give results which are well correlated with the degree of oxidized flavour.

In the present investigation the ferric thiocyanate peroxide technique has been applied to a study of the oxidation at 2–5° C. of milk fat in its natural globular state in whole milk, in artificial emulsions and in the form of clear filtered fat. The results have shown that, contrary to the accepted belief, copper-induced oxidized flavour in whole milk is derived partly from oxidative changes in the milk fat itself. During the course of the

investigation it further became evident that oxidation of an unidentified component of the skim milk phase contributes substantially to this type of off-flavour in whole milk. The results of experiments on which the latter conclusion is based are given in Part II of this paper.

METHODS

Flavour judgement and grading. The methods used were very similar to those described in a previous investigation (7). The same panel of four laboratory workers was employed. Points were allotted as follows:

0 Absence of off-flavour.
1 A very slight off-flavour.
2 A slight but well defined off-flavour.
3 A strong off-flavour.
4 A very strong off-flavour rendering the milk quite unacceptable as a beverage.

The graders were unaware of the identity of the samples. Their results were recorded independently and then averaged to the nearest $\frac{1}{4}$ point. In two major experiments the grading tests were repeated during the day to give three replicates for statistical analysis. On these occasions the samples were resubmitted under different code numbers, and the graders did not confer until the series was completed. In these two experiments the grade scores (the mean of twelve results) are given to two decimal places to permit a more accurate presentation of the significance of differences between means. Statistical treatment of other grading tests was not provided for, since it was considered that with the same grading panel dealing with the same type of off-flavour throughout, the grading error would be fairly constant. In the two experiments analysed the standard error of the mean of four results was approximately $\frac{1}{4}$ point, and the differences required for significance between two means were approximately $\frac{3}{4}$ point and 1 point at the 5 and 1% levels respectively.

In a few instances 'triangular' tasting tests were employed and the results analysed according to the method of Roessler, Warren & Guymon (10), who have discussed this method of testing.

Determination of fat peroxide values. The peroxide values of milk fat were determined by the method of Loftus Hills & Thiel (6). Fat was obtained from milk by centrifuging in 250 ml. lots in a Universal centrifuge. The skim milk was discarded, sufficient cold distilled water added to dilute the cream layer to about 30% fat and the cream churned in the centrifuge bottle in a mechanical shaker. The butter grains were melted at 45° C. and the fat filtered through a no. 540 filter-paper. 0·3 ml. fat was taken for each determination. Colour intensities were measured with an Evelyn photoelectric colorimeter using filter number 515. Peroxide values are expressed as milli-equivalents oxygen/kg. fat.

Determination of ascorbic acid. Ascorbic acid was estimated by the phenol-indo-2:6-dichloro-phenol titration method of Sharp (11).

Determination of copper. Copper was estimated in milk fat using the acid extraction technique described by McDowell (12).

Determination of phosphorus in milk fat. The fat (0·1 ml.) was ashed in 1:1 H_2SO_4 (1 vol. conc. H_2SO_4:1 vol. water) and 30% H_2O_2 at 160–180° C. The H_2O_2 was added dropwise until the mixture was water-clear. Phosphorus was then estimated as phospho-

molybdate blue, using 1:2:4-amino-naphthol-sulphonic acid as a reducing agent. The colours were measured with an Evelyn photo-electric colorimeter using filter number 660.

Determination of α-tocopherol. α-Tocopherol in milk fat was estimated by the method of Quaife (13).

Source and method of treatment of milk samples. The milk used was fresh morning milk taken on different occasions from one of several herds supplying market milk to the Melbourne metropolitan area. The milk was pasteurized at 145° F. for 30 min. immediately on arrival at the laboratory. Where milk was treated with copper the metal was added as an aqueous solution of $CuCl_2$ after pasteurization. Milk samples were stored in 1, 2 or 3 l. quantities in parchment-sealed Erlenmeyer flasks in a refrigerator at 3° C. for periods of up to 1 week. Every precaution was taken to protect samples from undue exposure to light.

EXPERIMENTAL PROCEDURE AND RESULTS

The effect of added copper on the flavour and fat peroxide values of pasteurized whole milk

Throughout the year it was necessary to add at least 0·5 and usually 1·0 p.p.m. of copper to pasteurized whole milk before a marked off-flavour could be consistently induced. Without added copper, very slight oxidized flavours developed only occasionally and then with more than 4 days' storage. With 0·1 p.p.m. of added copper the off-flavour was slight and irregular in occurrence. Attempts to increase the susceptibility of milk samples to oxidized flavour development by preliminary partial oxidation of the ascorbic acid with H_2O_2 or by exposure to daylight in the manner described by Krukovsky & Guthrie (14,15) were unsuccessful. Experiments dealing with the effect of light on the development of oxidized flavours in milk have already been described (Pont (7)).

Table 1. *Effect of added copper on oxidized flavour development and fat peroxide values in whole milk*

	Days stored at 3° C. 1			2		
Treatment	Fat peroxide value	Mean grade score	and comments	Fat peroxide value	Mean grade score	and comments
Control	0·02	0	Clean flavour	0·03	0	Clean flavour
Cu added, 0·1 p.p.m.	0·02	0	Clean flavour	0·04	0·72	Very slightly cardboard
Cu added, 1·0 p.p.m.	0·02	0	Clean flavour	0·05	1·17	Very slightly oxidized, cardboard
Cu added, 5·0 p.p.m.	0·02	0	Clean flavour	0·22	3·06	Strong cardboard, oily metallic
	4			6		
Treatment	Fat peroxide value	Mean grade score	and comments	Fat peroxide value	Mean grade score	and comments
Control	0·03	0·11	Clean flavour	0·03	0·11	Clean flavour
Cu added, 0·1 p.p.m.	0·05	1·17	Very slightly cardboard	0·05	1·22	Very slightly cardboard
Cu added, 1·0 p.p.m.	0·19	2·67	Oxidized, cardboard	0·41	3·67	Strong cardboard, oily metallic
Cu added, 5·0 p.p.m.	0·73	3·78	Very strong cardboard, oily metallic	1·31	4·00	Very strong cardboard, oily metallic

The minimum difference required for significance between any two means in the same day's grading is 0·50 point at the 5% level and 0·67 point at the 1% level.

The susceptibility of milk to oxidized flavour development has been reported by Krukovsky, Loosli & Whiting(16) to be associated with low levels of tocopherol in the milk fat. Tocopherol determinations were not consistently made in the present investigation, but a few analyses showed tocopherol values higher than 50 mg./kg. fat, indicating, on the basis of the latter authors' results, a high level of flavour stability.

Table 1 summarizes the results of a typical experiment and shows the relationship between concentration of added copper, intensity of off-flavour and fat peroxide values over a 6-day period at 3° C. The ascorbic acid content of the milk was 10 mg./l. It will be observed that there was a well-defined correlation between fat peroxide values and mean grade scores. Definite and objectionable oxidized flavours, usually described as oily, tallowy and cardboard, were always associated with fat peroxide values of 0·20 and higher. With fat peroxide values of about 0·10 and lower the intensity of off-flavour when present was slight, and the emphasis in the comments was on the cardboard element in the flavour. The fat peroxide value of untreated milk was always 0·02 or less when fresh and did not rise to more than 0·05 after 7 days' storage.

The flavour of whole milk reconstituted from fresh skim milk and oxidized milk fat

The association of oxidized flavour with measurable fat peroxide formation suggested that oxidation reactions in the fat might be in part responsible for the flavour. The nature and intensity of off-flavour in milk reconstituted from fresh skim milk and oxidized milk fat was therefore determined. Care was taken to ensure that the fat used was oxidized under conditions comparable with those observed for milk in the previous experiments. The fat was separated from pasteurized milk by separating, churning and melting the butter grains at 45° C. The liquid fat was centrifuged and clarified with a dry pulp filter prepared from acid-washed filter-paper. In this way quantities of fat could be produced of the order of 500 g. with a peroxide value of 0·02. Analyses of different batches of fat for phosphorus gave negative results indicating a negligible phospholipin content.

A small amount of copper was added by standing the fat in contact with clean copper turnings for 15 min. at 45° C. and then carefully decanting. Analyses showed values between 0·1 and 0·2 p.p.m. copper in the fat after this treatment. Such fat was found to oxidize at about the same rate as the fat in whole milk containing 1·0 p.p.m. copper. By diluting fat freshly exposed to copper with control fat and storing for a week at 3° C. a series of samples was obtained with peroxide values ranging up to 0·50. These were reconstituted with fresh pasteurized skim milk by means of a small hand-operated homogenizer, the fat percentage being adjusted to 4·0, and graded immediately. The initial fat content of the skim milk was usually 0·05% and never higher than 0·06% by the butyl alcohol modification of the Babcock test. The results of the tasting tests are shown in Table 2.

Table 2. *Nature and intensity of off-flavour in whole milk reconstituted from fresh skim milk and oxidized milk fat*

Fat peroxide value	0·02	0·08	0·15	0·25	0·43
Mean grade score (points)	0·31	0·52	0·81	2·33	2·33
Comments	Flat	Stale	Slightly oily?	Oily and metallic	Oily and metallic

The minimum difference required for significance between two of the means given in the table is 0·37 point at the 5% level and 0·50 point at the 1% level.

This experiment was repeated with substantially similar results. In both cases well-defined oily and metallic flavours were observed in the reconstituted milks with fat peroxide values of 0·25 and above. Highly significant differences in grade score were observed between milk made from control fat (P.V. 0·02) and fat oxidized to a P.V. level of 0·15, though in this sample the off-flavour was subtle and more difficult to define. The off-flavour observed in the reconstituted milks was not identical with the typical copper-induced oxidized flavour of normal whole milk. It had a similar oily-metallic character but the flavour entity commonly known as cardboard or cappy, so typical of the defect in normal whole milk, was absent from the reconstituted milks.

In experiments involving the reconstitution of oxidized milk fat with skim milk, Krukovsky, Ellis & Barnes(17) reported that an oxidized flavour developed in the milk within a few minutes of mixing and rapidly intensified. In the present investigation no differences were observed in the nature and intensity of off-flavour over a period of several hours after reconstitution, and there was no evidence that the off-flavour was due to interaction between oxidized fat and other milk constituents. 'Triangular' tasting tests (p. 317) carried out on similarly oxidized fat in bulk confirmed that the oily flavour noticed in the reconstituted milk was derived from the fat itself. At a peroxide value of 0·35 the oily metallic flavour was clearly evident in the fat. Fat with a peroxide value of 0·2 could also be differentiated by taste from control unoxidized fat at a high level of significance ($P = 0{\cdot}01$).

Copper-induced oxidation of milk fat in artificial emulsions

To provide physical conditions for the oxidation of the fat more closely resembling those in whole milk, milk fat was oxidized in emulsified form in a synthetic milk. The synthetic milk was prepared by the method of Clark(18). The casein used was acid-precipitated from fresh skim milk, washed, and redissolved immediately without being dried. Fresh unoxidized milk fat was dispersed in the synthetic milk with a small hand-operated homogenizer to give a fat concentration of 4%. Microscopical examination showed the fat globules ranged in diameter from less than 1 μ to about 10 μ, most being 4–5 μ. Copper was added at the rate of 1·0 p.p.m. and the 'milk' was then stored for 1 week at 3° C. Regular examinations were made for off-flavour and fat-peroxide values. The synthetic milk had a flat salty taste which made discernment of off-flavour difficult.

Table 3. *Rate of oxidation and intensity of off-flavour of milk fat in a synthetic milk*

		Days stored at 3° C.					
		3		5		7	
No.	Treatment	Fat peroxide value	Grade score and comments	Fat peroxide value	Grade score and comments	Fat peroxide value	Grade score and comments
1	Control	0·03	Flat, salty	0·03	Flat, salty	0·04	Flat, salty
2	Fat from 1 re-dispersed in fresh skim milk	—	0 Clean flavour	—	0 Clean flavour	—	0 Clean flavour
3	1·0 p.p.m. added Cu	0·15	Watery, stale	0·30	Stale, oxidized?	0·47	Stale, oxidized?
4	Fat from 3 re-dispersed in fresh skim milk	—	1 Stale, slightly oxidized	—	2 Oily, tallowy	—	2½ Oily, tallowy

For grading, therefore, subsamples were prepared on each occasion by centrifuging off the fat as cream and redispersing it in fresh skim milk. The results are summarized in Table 3.

The results of this experiment confirmed those reported in the previous section. The rate of fat oxidation was similar to that observed in normal whole milk containing 1·0 p.p.m. added copper. At a fat peroxide level of 0·30 the synthetic milk had an off-flavour which, though difficult to describe, clearly distinguished it from the control. When the fat was redispersed in skim milk the oily nature of the defect was more clearly evident. At a peroxide level of 0·15 the synthetic milk could not be distinguished with certainty from the control, but an oxidized type of off-flavour was apparent in the sub-sample prepared from skim milk. No off-flavours were produced by the action of 1·0 p.p.m. copper in synthetic 'skim milk', i.e. synthetic milk to which fat was not added.

DISCUSSION AND CONCLUSIONS

Thurston *et al.* (2) and Nelson & Dahle (19) have previously compared the flavour of milk reconstituted from fresh skim milk and oxidized milk fat, with the copper-induced oxidized flavour of normal whole milk, but their results were conflicting. Furthermore, no useful indication of the source of the defect in whole milk was given since the milk fat used in their experiments was previously heated at 60° C. or higher until a marked tallowy flavour appeared. It is probable that under these conditions, which are radically different from those favouring the development of the defect in milk, different end-products and flavours would appear in the fat.

Our experiments have shown that well-defined copper-induced oxidized flavour in whole milk is always accompanied by measurable peroxide formation in the milk fat, roughly proportional to the intensity of the off-flavour. Furthermore, milk fat, oxidized externally to milk in both the solid and emulsified states to peroxide values of the same order and at comparable temperatures, exhibits an oily-metallic off-flavour, both in itself and when redispersed in fresh skim milk. The results justify the conclusion that oxidation reactions in milk fat play a significant part in the development of oxidized flavour in whole milk, and contribute an oily-metallic characteristic to the defect.

PART II. THE OCCURRENCE AND SIGNIFICANCE OF OXIDIZED FLAVOUR IN SKIM MILK

On surveying the literature on the subject of oxidized flavour, it appears that skim milk is not susceptible to this defect. Thurston *et al.* (2) refer to a general belief that oxidized flavour has never been known to occur in skim milk, and neither Brown & Thurston (20) nor Greenbank (21) in their reviews on oxidized flavour deal with the occurrence of the defect in this product. Resistance by skim milk to the development of oxidized flavour would be consistent with the earlier theory that the flavour was due to fat oxidation, and with the present conception that it is derived from oxidation of phospholipin in the fat-globule membrane. However, in the few papers where experiments dealing with the effect of copper in skim milk have been reported the results do not justify the conclusion that skim milk is immune or even resistant to oxidized flavour. Both Tracy & Ruehe (22) and Roland & Trebler (23), for instance, reported that skim milk contaminated with copper developed a metallic flavour, though the authors distinguished it from the typical oxidized flavour of whole milk. The results of Thurston *et al.* (2) indicate that skim milk, prepared from whole milk with oxidized flavour, retains some of the defect. Trout & Gould (24)

reported that while homogenization inhibited the development of oxidized flavour in copper-contaminated whole milk, the process was ineffective in skim milk. Swanson & Sommer(25) found that with 2 p.p.m. copper, both skim milk and whole milk developed the same intensity of oxidized flavour. Nelson & Trout(26) stated that skim milk may frequently have an off-flavour which suggests that of paper, but advised that it should not be confused with the oxidized flavour of whole milk.

The lack of agreement shown in these reports is probably due to differing interpretations of the term 'oxidized flavour'. If the oxidized flavour of whole milk contained flavour elements derived from or associated with the fat one would not expect the defect to be reproducible in its entirety in skim milk; also, metallic, papery and similar types of flavours according to the original usage of Guthrie, Roadhouse & Richardson(27) are oxidized flavours, whether they occur in skim milk or whole milk, and are rightly described in that way.

In Part I it was shown that an oily-metallic component in the oxidized flavour of whole milk was derived from oxidation of the milk fat. Milk reconstituted from fresh skim milk and oxidized milk fat exhibited this oily-metallic characteristic, but lacked the cardboard type of off-flavour commonly associated with the defect in normal whole milk. This suggested that the compound responsible for the cardboard element in the flavour was derived from reactions in the constituents of the milk other than fat. The effect of added copper on the flavour and odour of skim milk was therefore investigated.

METHODS

The methods of grading and of analysis, and also the source and method of treatment of the milk samples are the same as in Part 1. The skim milk used was obtained by separating pasteurized whole milk in a Lister 25 gal./hr. separator at 40° C. The fat content of the skim milk by the butyl alcohol modification of the Babcock test was usually 0·05% and never higher than 0·06%.

EXPERIMENTAL PROCEDURES AND RESULTS

The effect of added copper on the flavour and odour of skim milk

With 1·0 p.p.m. copper added after pasteurization a pronounced off-flavour and odour usually developed in the skim milk after 2 or 3 days' storage at 3° C. The flavour was slightly astringent, and the odour had a very slight drying-oil characteristic resembling that of some types of printed cardboard. Allowing for the inherent lack of body and richness in skim milk, the flavour had a marked resemblance to that produced by copper in the earlier stages of its action in whole milk. It appeared, in fact, to supply all of the cardboard element in the oxidized flavour of whole milk. The flavour in skim milk therefore is referred to in this paper as cardboard flavour, in spite of the common use of that term to describe the more complex oxidized flavour of whole milk.

With 5·0–10·0 p.p.m. copper the cardboard flavour appeared usually after 1 day at 3° C. and rapidly became intensified. With copper concentrations above 10·0 p.p.m. a very intense off-flavour and aroma rapidly appeared which, though it resembled the cardboard defect, was different in character. On several occasions in the early spring skim-milk samples were found to be resistant to the development of the cardboard defect with 1·0 p.p.m. copper, though they were susceptible to higher concentrations. With 0·1 p.p.m. copper the flavour was absent or very slight.

The effect of ascorbic acid on the development of off-flavour in skim milk, whole milk and washed cream

When a sample of skim milk failed to develop a cardboard flavour with 1·0 p.p.m. copper it was found that the defect could be readily induced if 50 mg./l. of ascorbic acid was added with the copper. Olsen & Brown (28) showed that an intense oxidized flavour could be induced in washed cream by the action of copper and ascorbic acid at this concentration. It was decided, therefore, to compare the effect of copper and varying concentrations of ascorbic acid on the flavour of skim milk, whole milk and washed cream.

Washed cream was prepared by diluting 45% cream tenfold in warm water and re-separating, this being repeated five times. The cream was finally diluted with water to give a product with 10% fat and was adjusted to pH 6·6 with a 0·2% sodium citrate buffer. 1·0 p.p.m. copper was added to samples of pasteurized skim milk, whole milk and washed cream, and the concentration of ascorbic acid in each adjusted by addition of the pure compound to give a range from 7·0 mg./l., which was the original concentration, to 500 mg./l. The samples were stored at 3° C. and graded after 4 days. The results are given in Table 4.

Table 4. *The effect of ascorbic acid on off-flavour in skim milk, whole milk and washed cream. Copper concentration* 1·0 *p.p.m.*

Sample	Ascorbic acid concentration mg./l.	Grading after 4 days at 3° C.	
		Grade points	Comments
Skim milk, no Cu	7·0	0	Clean flavour
Skim milk, 1·0 p.p.m. Cu	7·0	2	Cardboard
	20·0	2¾	Strong cardboard
	50·0	3	Strong cardboard
	100·0	3½	Strong cardboard
	200·0	½	Cardboard?
	500·0	0	Clean flavour
Whole milk, no Cu	8·0	0	Clean flavour
Whole milk, 1·0 p.p.m. Cu	8·0	1	Very slightly cardboard
	20·0	2½	Cardboard and oily
	50·0	3½	Strong cardboard and oily
	100·0	2½	Cardboard and oily
	200·0	½	Cardboard?
	500·0	0	Clean flavour
Washed cream, no Cu	7·0	0	Clean flavour
Washed cream, 1·0 p.p.m. Cu	7·0	0	Clean flavour
	20·0	2½	Linseed-oil flavour
	50·0	4	Linseed-oil flavour
	100·0	4	Linseed-oil flavour
	200·0	2	Linseed-oil flavour
	500·0	0	Clean flavour

The odour and flavour of the skim milk and whole milk when graded were strongly cardboard in character, the whole milk possessing in addition the unpleasant oily flavour characteristic of the defect in this product. The off-flavour of the washed cream was different from that of the skim and whole milk. Some members of the grading panel compared the odour to that of new-mown grass; others thought that it resembled that of linseed oil. This off-flavour in the washed cream either concentrated or dilute was not recognized as a component of the normal oxidized flavour of whole milk as observed in this laboratory.

In control samples containing ascorbic acid but no copper no off-flavours developed in the skim and whole milk. The off-flavour in the washed cream, however, developed with equal intensity in spite of the absence of copper. The development of the off-flavour was prevented entirely by the addition of 3% casein or by reconstitution of the washed cream in skim milk.

The effect of copper on the flavour of skim milk, buttermilk, and skim milk containing added buttermilk

The off-flavour in whole milk induced by copper is generally attributed to oxidation of phospholipin. Some experiments were therefore conducted to determine whether the intensity of cardboard flavour in skim milk was affected by variations in the phospholipin content. Comparisons were made between the effects of copper on the flavour of normal skim milk, skim milk obtained by centrifuging cold whole milk, buttermilk, and skim milk containing buttermilk added in sufficient quantity to restore the phospholipin content of the original whole milk. The centrifuged milk was prepared by allowing pasteurized whole milk to stand for 24 hr. at 3° C. and then centrifuging it at 2000 r.p.m. for 30 min. The resultant skim milk, after removal of the supernatant solid cream layer, had a fat content of 0·20%. As the fat content of these products was higher than that of normal skim milk, control samples of skim milk were provided in which the fat content was adjusted to comparable levels by the addition of cream. 1·0 p.p.m. of copper was added to the samples which were held at 3° C. and graded after 2 and 5 days.

The results which are given in Table 5 show that increasing the phospholipin content of skim milk by the addition of buttermilk had no significant effect on the intensity of cardboard flavour produced by copper. Actually the off-flavour in the buttermilk itself was no stronger than that in the skim milk. The method of centrifuging milk would be expected to remove from the skim milk as completely as possible those compounds normally adsorbed on the fat globule, but this had no effect on the degree of off-flavour developing. The intensity of off-flavour induced by copper in whole milk appeared to be slightly less than that in the skim milk.

Table 5. *The effect of* 1 *p.p.m. copper on flavour of gravity separated skim milk, buttermilk and skim milk containing added buttermilk*

Sample no.	Treatment	Grade score points and comments: 2 days	Grade score points and comments: 5 days
1	Skim milk, control	0 Clean flavour	0 Clean flavour
2	Sample 1 plus 1·0 p.p.m. Cu	0 Clean flavour	2½ Cardboard
3	Skim milk, fat 0·20%	0 Clean flavour	0 Clean flavour
4	Sample 3 plus 1·0 p.p.m. Cu	0 Clean flavour	2½ Cardboard
5	Skim milk plus 15% buttermilk, fat 0·20%	0 Clean flavour	0 Clean flavour
6	Sample 5 plus 1·0 p.p.m. Cu	0 Clean flavour	2½ Cardboard
7	Buttermilk, fat 1·0%	½ Off-flavour?, not oxidized	¾ Off-flavour?, not oxidized
8	Sample 7 plus 1·0 p.p.m. Cu	1 Slightly cardboard	2½ Cardboard and slightly oily?
9	Cold-centrifuged skim milk, fat 0·20%	0 Clean flavour	0 Clean flavour
10	Sample 9 plus 1·0 p.p.m. Cu	½ Cardboard?	2 Cardboard
11	Whole milk plus 1·0 p.p.m. Cu	0 Clean flavour	1¾ Cardboard, slightly oily

Observations on physical and chemical properties of compound responsible for cardboard flavour

Three litres of pasteurized skim milk containing 5·0 p.p.m. copper were stored at 3° C. for 7 days, by which time a very strong cardboard odour and flavour had developed. The milk was then concentrated at 60 mm. pressure to two-thirds of its original volume, using all-glass equipment and an ice-cooled condenser. The distillate, approximately 1 l., had the typical cardboard odour to an intense degree. The concentrate was practically free of the odour or flavour. When the distillate was added to fresh skim milk at the rate of 5–10% it imparted a characteristic cardboard odour and flavour. Such milk was graded from $2\frac{1}{2}$ to $3\frac{1}{2}$ points and was indistinguishable by taste and odour from skim milk in which the off-flavour had developed normally.

The compound responsible for the cardboard odour was soluble in light petroleum and could be extracted with this solvent from the distillate. The compound was also removed from the distillate by adsorption on activated charcoal, from which it could be freed by steam distillation at 100° C. It could be concentrated by redistilling the distillate at 100° C., practically all the odour coming over with the first 30%. The cardboard odour in the distillate was destroyed by strong reducing and oxidizing agents (nascent H and $KMnO_4$) and was not affected by less intense treatment (3% H_2O_2, glutathione and ascorbic acid). It was stable to weak acids, but on treatment with alkali gave a strong soap-like odour.

Distillates from oxidized whole milk and oxidized skim milk were identical, as far as could be determined by flavour and by the simple chemical tests mentioned. Both distillates when added to fresh skim milk or whole milk gave the same off-flavour. The off-flavour induced by ascorbic acid in washed cream was found to be steam volatile, and a distillate from an affected sample of this product was also examined. While the odour of the washed cream distillate resembled that of the skim milk and whole milk distillates, the cardboard element in it was much less pronounced. It was dominated by the same linseed-oil characteristic which was so evident in the affected cream itself.

An attempt was made to isolate the compound responsible for cardboard flavour by distilling at 60 mm. pressure 12 l. of affected skim milk. The 3 l. distillate were further distilled at 100° C. to give 1 l. concentrated distillate. This was extracted with purified light petroleum (B.P. below 40° C.) which was then evaporated at 40° C. A few milligrams of a colourless oily liquid were obtained which exhibited the cardboard odour very strongly. The quantity was too small to permit purification and further examination. Larger scale distillations are now being undertaken.

DISCUSSION AND CONCLUSIONS

The cardboard flavour described here is probably identical with the emery flavour of whole milk referred to by Mohr & Wellm (29). These authors distinguished the latter from rancid, oily and tallowy flavours in milk, and reported that the causative compound was steam-volatile and soluble in fat. Their observations and the evidence presented here justify the conclusion that copper-induced oxidized flavour in whole milk consists of at least two distinct flavour elements. One is an oily-metallic flavour due to oxidation of the milk fat, and another is a cardboard flavour arising from a reaction or series of reactions which proceed with equal effect in skim milk and whole milk.

This conclusion is not readily reconciled with the theory that oxidized flavour in whole milk is due to oxidation of phospholipin. It is reasonable to assume that both the total quantity of phospholipin and the ratio of membrane phospholipin to dispersed phospholipin is less in skim milk than in whole milk. If cardboard flavour is due to reactions in globule membrane phospholipin only, one would not expect the flavour to occur with equal intensity in skim milk and whole milk. If the flavour is caused by phospholipin dispersed in the milk serum one would expect it to be affected by variations in the concentration of phospholipin in this form; evidence has been given that this is not so. In addition, the effects of homogenization, agitation and alternate freezing and thawing of milk in preventing oxidized flavour have hitherto been explained by assuming that phospholipin freed from the fat globule by these mechanical treatments does not give rise to the flavour.

The work of Olsen & Brown (28) in showing that washed cream, in the presence of copper and ascorbic acid, develops an intense oxidized flavour has been accepted as further proof that the defect in whole milk is associated with the fat-globule membrane. In the present investigation, however, obvious differences between this flavour in washed cream and the flavour induced by copper in whole milk were noted. In addition, it was observed that the flavour in the washed cream differed from that in whole milk in occurring quite readily in the absence of copper. It was also readily prevented by the restoration of casein to the washed cream. Both the latter observations were made also by Forster & Sommer (30). The relationship of this off-flavour in washed cream to normal oxidized flavour is not clear, but these observations suggest that it plays no important part in the overall defect in whole milk.

SUMMARY

With 1·0 p.p.m. of added copper, well-defined oxidized flavours described as cardboard, oily and metallic developed in pasteurized whole milk after 2–3 days' storage at 3° C. With 0·1 p.p.m. copper only slight and irregular off-flavours, usually described as cardboard, appeared. Partial oxidation of the ascorbic acid of the milk with H_2O_2 did not increase its susceptibility to oxidized flavour development.

Marked off-flavours were always associated with peroxide values in the milk fat of 0·2 and more (m.equiv. oxygen/kg.) determined by the ferric thiocyanate technique. With fat peroxide values of about 0·5, usually obtained after 6–7 days at 3° C. with 1·0 p.p.m. copper, the oxidized flavour was very strong and the milk usually undrinkable.

Filtered milk fat, free of phospholipin and obtained from fresh pasteurized milk, was oxidized externally to milk both in the solid and emulsified states with copper at 3° C. When reconstituted with fresh skim milk it exhibited a slight off-flavour at a peroxide value of from 0·15 to 0·20 and an oily-metallic flavour at 0·30–0·35. At the latter peroxide value the oily metallic flavour was perceptible when the fat itself was tasted. The reconstituted milks lacked the cardboard flavour commonly encountered in the oxidized whole-milk samples.

A cardboard flavour, identical with the cardboard component of oxidized flavour in whole milk, was produced by the action of copper in pasteurized skim milk. It was usually well developed after 3 days at 3° C. with 1·0 p.p.m. copper. The simultaneous addition of 50 mg. ascorbic acid/l. intensified the flavour in skim milk and whole milk, while 200 mg. ascorbic acid/l. or more prevented its development.

The cardboard flavour developed to the same extent in buttermilk as in skim milk,

and was unaffected by treatment which altered the phospholipin content of the skim milk. It is unlikely therefore that phospholipin is the source of the flavour. The compound responsible for the flavour is steam volatile, and can be isolated from affected skim milk and whole milk by distillation under reduced pressure and extraction of the distillate with light petroleum.

The flavour differed in important respects from the oxidized flavour in washed cream produced by addition of ascorbic acid.

It is concluded that this cardboard flavour and the oily-metallic flavour of oxidized fat largely constitute the oxidized flavour defect in whole milk.

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637.127.7

488. AN IMPROVED METHOD OF OBSERVING THE CLOTTING OF MILK CONTAINING RENNIN

By N. J. BERRIDGE

National Institute for Research in Dairying, University of Reading

(With 1 Figure)

The method of assaying rennin described by Berridge(1) requires the formation under reproducible conditions of a thin flowing film of milk. Although the skill needed to keep the film flowing at the optimum rate as the clotting point is approached is readily acquired, an automatic method has the advantage of complete reproducibility. A suitable film can be maintained if 10 ml. of milk are placed in a boiling tube of 2·2 cm. internal

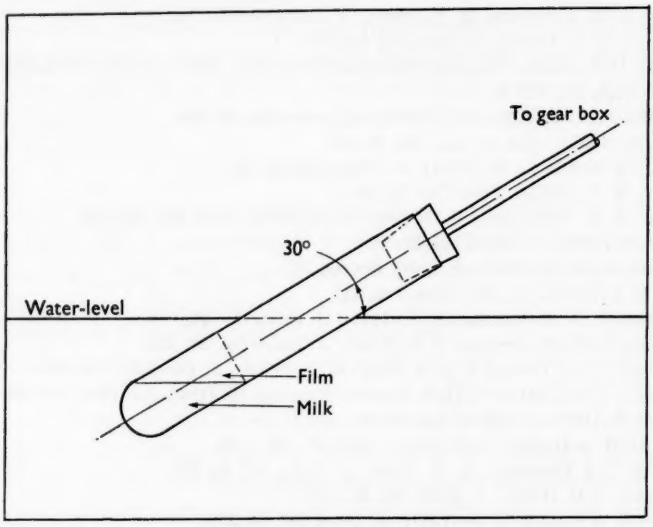

Fig. 1. Position of rotating tube in water-bath.

diameter and the tube, with its axis inclined at about 30° to the horizontal, rotated at 3·0 r.p.m. In the apparatus now used, the boiling tube is mounted on a rubber bung, which in turn is mounted concentrically on a spindle connected through Meccano gears to a 'Sangamo' electric clock motor. A special mechanical coupling allows the bung to be rotated by hand one revolution forwards, thus allowing 20 sec. for fitting or releasing the boiling tube before the drive is again taken up.

The tube is immersed in the water of the thermostat to such a depth that the film of milk is always below the surface and clotting is observed from above, or from the side. The arrangement of the apparatus will be clear from the diagram.

Several months' use have shown unequivocally that reproducible clotting times are easier to achieve with this apparatus than by the old method.

The author wishes to thank Mrs C. Woodward for technical assistance.

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637.17:636.4

489. THE PHYSICAL PROPERTIES OF SOW'S MILK AS A FUNCTION OF STAGE OF LACTATION

By W. G. WHITTLESTONE

Ruakura Animal Research Station, Department of Agriculture, Hamilton, New Zealand

(With 4 Figures)

As the literature on the physical properties of sow's milk is somewhat meagre, and because of the ready availability of such milk following the introduction of routine machine milking at this Station (1), the following study was undertaken. Fundamental studies on the nature of the milk-ejection process in the sow require such information as the viscosity and density of sow's milk, so that the work has a twofold value. The properties density, viscosity, electrical conductivity and hydrogen-ion concentration were determined throughout the lactation of ten sows, while the properties of colostrum were studied for two animals. The distribution of the fat globules is described elsewhere (2).

METHODS

Density. All densities were determined with a pyknometer of a volume equivalent to 10·1574 g. of water at 25° C. All determinations were carried out in a thermostat at 25° C. ± 0·05. The pyknometer was filled at room temperature (below 25° C.), placed in the thermostat, and the excess milk from the capillary ends wiped away. After coming to equilibrium the instrument was removed from the thermostat, cooled and dried by washing with anhydrous acetone, thus causing a contraction of the contents and avoiding spillage. It was then weighed at room temperature.

Viscosity. An Ostwald viscometer was used for this determination. A standard 40% sucrose solution of viscosity 5·206 centipoises (*International Critical Tables*) and density at 25° C. of 1·1774 was used for calibration. This gave a mean viscometer time of 107·65 sec. All determinations were carried out at 25° C. in the thermostat used for the density determinations. All values are given in centipoises, except a few determinations on colostrum for which density figures were not available. Readings were reproducible to better than ± 0·1 sec. From the above figures the viscometer constant is seen to be

$$C = 5{\cdot}206/1{\cdot}774 \times 107{\cdot}65$$
$$= 0{\cdot}04108.$$

For a time t and a density ρ the viscosity in centipoises is given thus: $\eta = C\rho t$.

Electrical conductivity. A Philips dipping cell unit was used for conductivity determinations. This cell consists of two black platinum electrodes held rigidly by a glass bridge within a robust protecting tube. The electrodes are vertical, which is essential for work with milk. The cell constant was 0·685, standardized against N/10-potassium chloride. Determinations were made in a thermostat at 25° C. ± 0·025. The cell resistance was determined with a Muirhead Universal bridge type A-154-A. The source of 3000 c.p.s. alternating current for the bridge was a 'home-made' R-C oscillator stabilized with

negative feed-back and driving a pentode power amplifier which was matched to the bridge with a variable impedance matching transformer. The null-point detector consisted of a two-stage pentode amplifier followed by a 'magic eye'. The whole apparatus

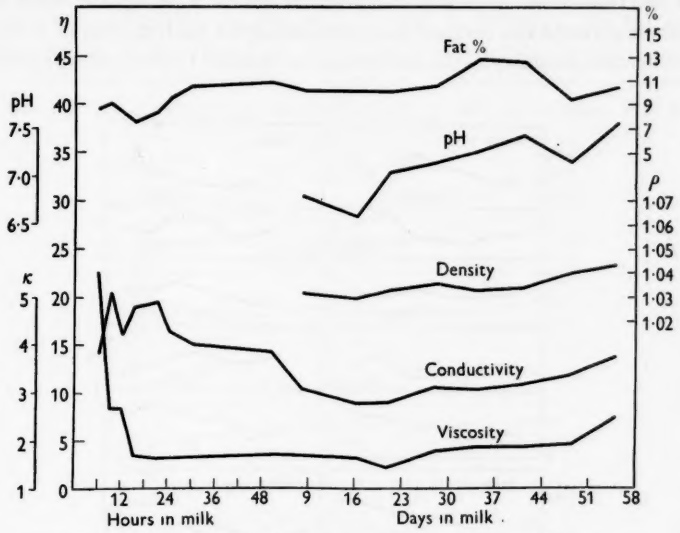

Fig. 1. Incomplete data for colostrum and milk from sow Flossie.

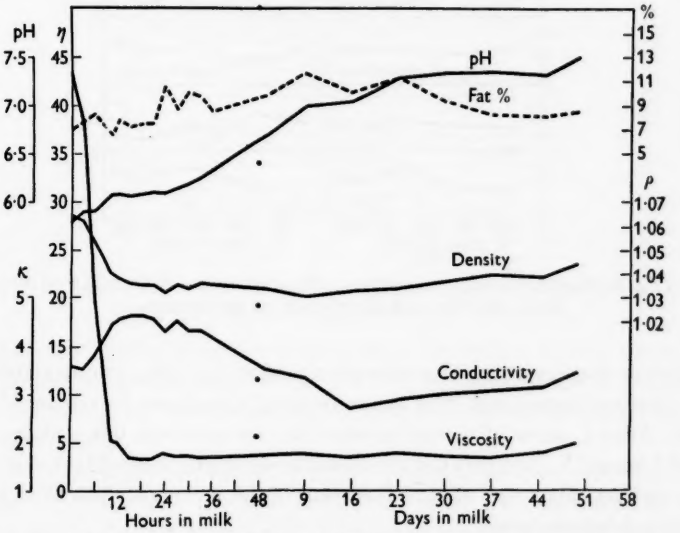

Fig. 2. Data for colostrum and milk from sow Florence.

was thoroughly screened and the bridge earthed via a modified 'Wagner earth'. Four-figure accuracy could be obtained with this equipment without difficulty. With milk trouble is sometimes experienced due to a fat layer forming on the electrodes. This was overcome by rinsing the cell with a dilute non-ionic detergent (Lissapol N). Because of

its non-ionic character and the fact that it is readily rinsed away, this method introduced no significant errors into the determinations.

Hydrogen-ion concentration. A Coleman electrometer was used with a glass electrode for the pH determinations. This was standardized against a phosphate buffer of pH 6·98. All readings are given to two decimal places and involve a reading error of ±0·01 pH unit. The electrochemical accuracy of the instrument is regarded by the makers as 0·05 pH unit.

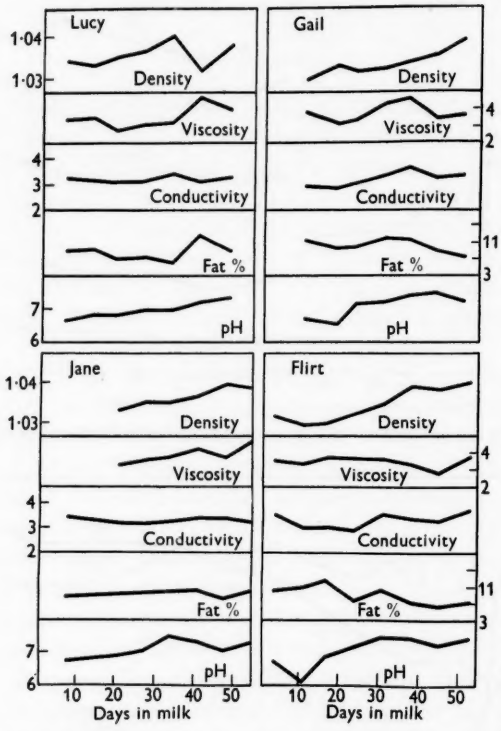

Fig. 3. Density, viscosity, conductivity, pH and fat content of the milk from sows Lucy, Gail, Flirt and Jane throughout one lactation.

The problem of cleaning a glass electrode when used with a fatty, proteinaceous substance like sow colostrum is very real. This was conveniently overcome by the use of a non-ionic detergent. After a series of determinations the electrode was left soaking in a dilute solution of Lissapol N. Not only did this clean the electrode thoroughly, but it so stabilized the electrode that only a very slight adjustment of the asymmetry potential was required in a month's determinations.

Tests on the effect of standing in the cooler or at room temperature on pH and conductivity were made but no significant change recorded. Hence, normally, physical determinations were carried out in the afternoon, the samples having been drawn between 9 and 10 a.m. The samples were held in a cooler until required.

RESULTS

Fig. 1 shows the incomplete data for colostrum and milk from one sow (Flossie), while in Fig. 2 the complete graphs for sow Florence are given. The most notable changes in the colostrum-milk transition are the enormous fall in viscosity, the rise in electrical conductivity and fall in density. Figs. 3 and 4 set out the data for the remaining eight sows whose milk was studied after the colostrum had disappeared. It is evident that density, viscosity and conductivity are influenced by fat percentage. The trends vary

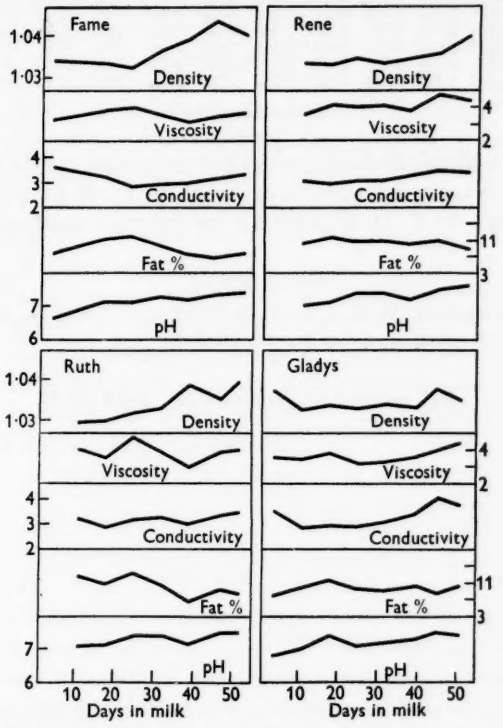

Fig. 4. Density, viscosity, conductivity, pH and fat content of the milk from sows Fame, Rene, Ruth and Gladys throughout one lactation.

from sow to sow, so that it is difficult to generalize. One of the clearest trends is that of pH, which increases throughout lactation. The fat percentage tends to fall, resulting in a rise in conductivity and density and fall in viscosity. However, these properties are more erratic than pH.

The average figures and the range for milk throughout lactation and excluding colostrum are set out in Table 1. No evidence of clinical mastitis appeared at any stage during the work, so that the figures may be taken as typical of normal sow's milk.

SUMMARY

The milk from ten sows has been studied over one lactation. The density, viscosity, electrical conductivity and pH have been recorded throughout the lactation, and in two cases the study extended to the colostrum.

Table 1. *Average figures for the density, viscosity, conductivity, pH and fat content of sow's milk during one lactation*

Sow	Density 25°/25° C.			Viscosity (centipoises)			Conductivity ($ohm^{-1} \times 10^6$)			Fat (%)			pH			No. of samples
	Mean	Max.	Min.	Mean	Max.	Min.	Mean	Max.	Min.	Mean	Max.	Min.	Mean	Max.	Min.	
Fame	1·0370	1·0436	1·0323	3·586	4·026	3·158	3·164	3·626	2·853	8·86	11·95	6·45	7·15	7·37	6·67	7
Rene	1·0350	1·0402	1·0330	4·157	4·427	3·655	3·198	3·513	2·943	10·68	11·95	8·95	7·32	7·64	7·00	7
Ruth	1·0342	1·0399	1·0294	3·909	4·801	2·987	3·250	3·538	2·906	10·35	13·40	6·65	7·33	7·52	7·10	7
Gladys	1·0346	1·0376	1·0326	3·677	4·490	3·204	3·356	4·100	2·945	9·78	11·70	7·85	7·24	7·55	6·82	8
Lucy	1·0360	1·0408	1·0321	3·553	4·782	3·118	3·246	3·483	3·108	8·81	13·10	7·15	7·04	7·36	6·78	7
Gail	1·0340	1·0396	1·0300	3·814	4·667	3·201	3·244	3·699	2·895	10·57	12·45	7·85	7·15	7·52	6·61	7
Jane	1·0366	1·0397	1·0333	3·974	4·943	3·320	3·349	3·493	3·236	9·41	10·45	10·20	7·16	7·52	6·75	7
Flirt	1·0349*	1·0407*	1·0296*	3·495*	3·777*	2·849*	3·352	3·812	2·944	9·14	12·65	6·50	7·11	7·52	6·16	8
Florence	1·0356	1·0441	1·0272	4·059	5·738	3·407	3·177	3·560	2·727	10·48	17·20	8·15	7·10	7·50	6·40	9
Flossie	1·0347	1·0434	1·0290	4·272	7·485	3·179	3·143	3·782	2·751	10·57	12·70	9·35	7·12	7·55	6·58	8

* Seven samples

The writer wishes to thank Mr D. M. Smith for permission to use the milk from lactating sows which were involved in his researches; Mrs D. R. Perrin for permission to use her butterfat figures; and Mr E. D. O'Reilly for his generous assistance in arranging for sampling the milk.

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637.17:636.4

490. THE DISTRIBUTION OF FAT-GLOBULE SIZE IN SOW'S MILK

II. THE INFLUENCE OF STAGE OF LACTATION

By W. G. WHITTLESTONE

Ruakura Animal Research Station, Department of Agriculture, Hamilton, New Zealand

(With 6 Figures)

In an earlier paper(1) the writer described the effects of stage of milking out on the distribution of fat globules in sow's milk. This paper described the method used for the determination of globule distribution and gave results for sows which had been in milk for periods ranging between 26 and 55 days. The present paper sets out the trends in fat-globule distribution for ten sows throughout lactation, two sows being followed at close intervals throughout the colostrum phase.

The accompanying figures give the pattern of globule size changes with advancing lactation. The variables time, size and percentage distribution of the size classes have been set out as a projection of a three-dimensional graph. The milk samples used for the determination of the fat-globule distribution are the same as those used for the physical properties study(2) previously published. In order to simplify correlating the two sets of data, the individual sow's names have been retained.

RESULTS

The two sows Flossie and Florence were studied throughout the colostrum phase as well as through the rest of the lactation. It should be noted that in the graphs setting out the results for these animals (Figs. 1, 2) the left-hand graph has a time scale in hours, while the right-hand one is in days. The remainder of the figures have a time scale in days. It is evident from the two sets of colostrum data that the globule size distribution is much narrower at the start of lactation, there being fewer large globules. Large globules increase in numbers until the transition to normal milk. No very obvious trends occur with advancing lactation. The vertical lines at the left of the distribution diagrams represent the height of the maximum; the higher this line the more nearly homogeneous the size distribution. Apart from colostrum having a somewhat narrower distribution than normal milk, no trends are evident.

One of the most noticeable changes occurring with the termination of the colostrum phase is the loss of a tendency to cream. Colostrum, despite its higher viscosity, creams readily, while normal sow's milk creams slowly. This change is accompanied by a loss of a tendency for the globules of fat to cluster, a change evident under the microscope.

It is evident from the data presented that the average size of the fat globule in sow's milk is smaller than that of cow's milk. The maximum frequency often occurs in the 0·5–1·0μ class and rarely exceeds the 2·0–2·5μ group. The factors influencing fat-globule size are little understood. The present data are of value only if regarded as further facts which may be fitted within a theoretical framework as our understanding of the mechanism of milk secretion extends.

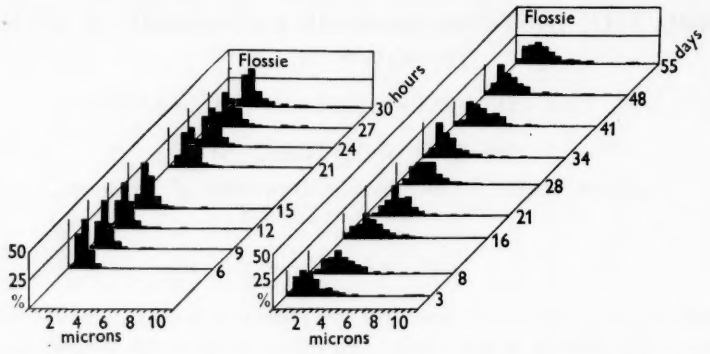

Fig. 1. The fat-globule distribution patterns throughout the colostrum phase and a normal lactation for sow Flossie

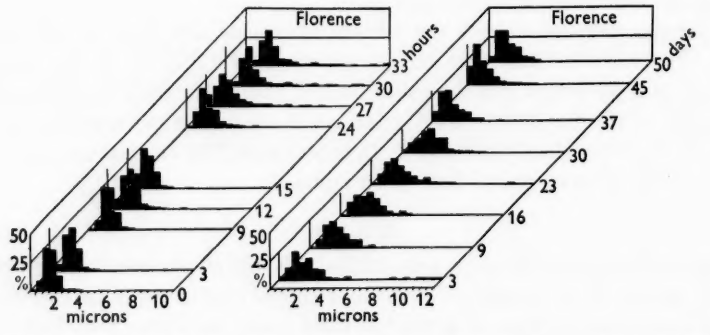

Fig. 2. The fat-globule distribution patterns throughout the colostrum phase and a normal lactation for sow Florence

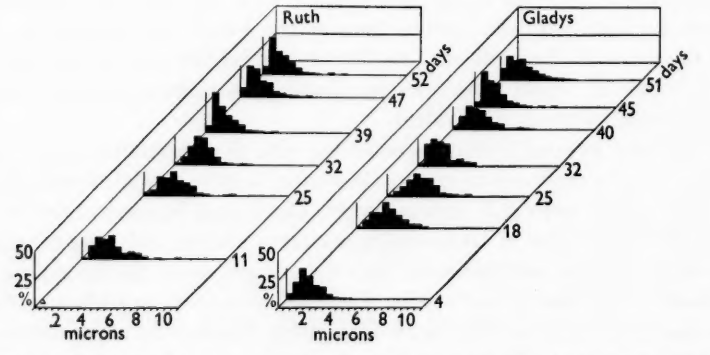

Fig. 3. The fat-globule distribution patterns throughout a normal lactation for sows Ruth and Gladys.

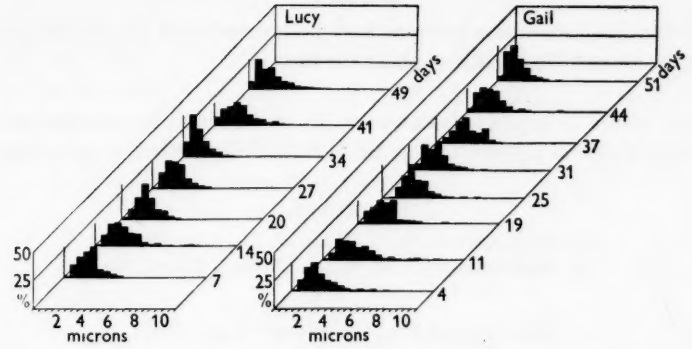

Fig. 4. The fat-globule distribution patterns throughout a normal lactation for sows Lucy and Gail.

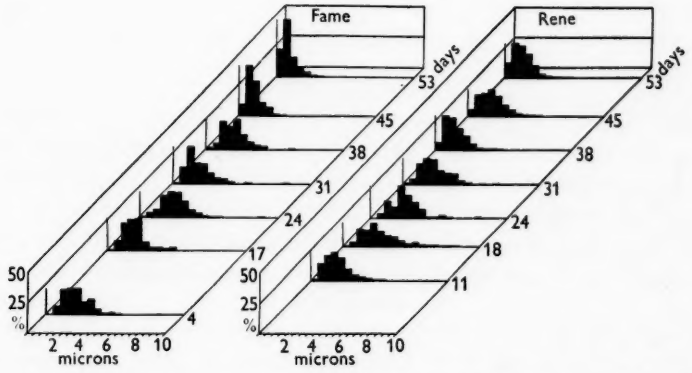

Fig. 5. The fat-globule distribution patterns throughout a normal lactation for sows Fame and Rene.

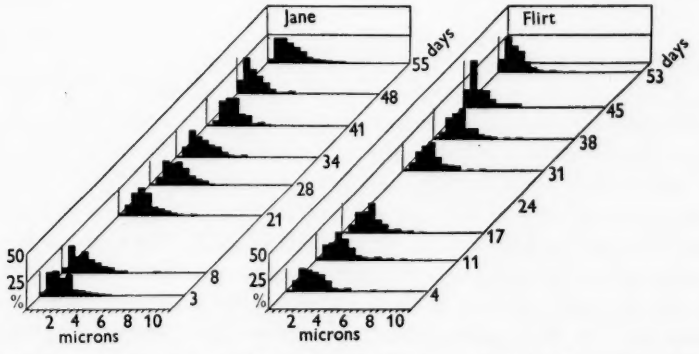

Fig. 6. The fat-globule distribution patterns throughout a normal lactation for sows Jane and Flirt.

SUMMARY

The fat-globule size distribution patterns have been determined throughout lactation for ten sows and through the colostrum phase for two.

The writer wishes to express his thanks to Mr D. M. Smith for permission to use the facilities of the Ruakura piggery, and to Mr E. D. O'Reilly for help in procuring samples.

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637.143.128

491. THE EFFECT OF PRE-HEATING MILK AT 230° F. (110° C.) ON THE KEEPING QUALITY OF SPRAY-DRIED WHOLE MILK

By J. C. D. WHITE and R. WAITE
The Hannah Dairy Research Institute, Kirkhill, Ayr,
H. B. HAWLEY
Messrs Aplin & Barrett Ltd., Yeovil,
J. G. CLARK
The A.P.V. Co. Ltd., London,
and KATHLEEN M. HENRY
National Institute for Research in Dairying, University of Reading

(With 2 Figures)

INTRODUCTION

Previous work(1,2,3) has shown that milk pre-heated at 190° F. before spray-drying produced a whole-milk powder of better keeping quality than when the pre-heating temperature was only 160° F. Moreover, increasing the processing temperature to 200° F. was no more beneficial than heating at 190° F. It has been assumed that the increased resistance to oxidative deterioration of powders made from milk pre-heated at the higher temperature resulted from thermal liberation of sulphydryl compounds which thereafter acted as antioxidants.

Recent developments in milk processing equipment have made possible the use of pre-heating temperatures higher than the atmospheric boiling-point of raw milk, and it seemed desirable to continue the earlier work by the use of a pre-heating temperature higher than 200° F. The present work is a comparison of the keeping and nutritional qualities of whole-milk powders prepared from milk pre-heated at 190 and 230° F.

EXPERIMENTAL

Heat treatment before drying

The general lay-out of the plant has already been shown in detail(1), and only two changes were made for the present experiments. The open heaters used previously to raise the temperature of the milk coming from the storage tanks to 110° F. were omitted, and this stage was carried out in the Scott tubular heater. The high-temperature pre-heating (230° F.) was done in an A.P.V., H.M. Paraflow Heat Exchanger placed between the Scott pre-heaters and the Kestner climbing film, three-pass evaporator. The connexions of the A.P.V. Heat Exchanger could also be arranged to heat the incoming milk (110° F.) regeneratively by counter-circulation with the outgoing milk (230° F.). The temperature of the incoming milk was raised to 140° F. in the regenerative section and finally to 230° F. in the second section of the heater. Regeneration caused a drop in temperature of the outgoing milk from 230 to 190° F. before it was fed to the evaporator. The difference

in heat treatment between the direct and regeneratively heated milks was thus to give a shorter time at 230° F. to the latter.

Since the powder manufactured from the milk pre-heated at 190° F. was to serve not only as a comparison with the powders from milk pre-heated at 230° F., but also as a link with the earlier experiments, it was essential to keep its heat treatment before drying as similar as possible to those earlier powders. This involved passing the 190° F. pre-heated milk to a balance tank immediately before the evaporator, whereas with the milk pre-heated directly to 230° F. this was obviously impracticable. The balance tank was therefore only used for the 190° F. milk, despite the variation this introduced to the length of heat treatment at the two temperatures.

To summarize, then, three whole-milk powders were manufactured from bulked milk from the same suppliers on three successive days. After being raised to a temperature of 110° F., the milk had the following heat treatments before evaporation and spray-drying.

Powder 1: heated from 110 to 190° F. and held in the hot well for approximately 5½ min. before being drawn into the evaporator through a control cock.

Powder 2: heated from 110 to 230° F., held at that temperature for approximately 22 sec. plus approximately 6 sec. during which the milk temperature was rising from 190 to 230° F., and fed direct to the evaporator at a pressure of 15 lb./sq.in.

Powder 3: heated from 110 to 230° F., held at that temperature for approximately 7 sec. plus approximately 14 sec. during which the milk temperature was between 190 and 230° F. (during heating and cooling), and fed direct to the evaporator at a pressure of 15 lb./sq.in.

The pre-heated milk was evaporated to a total solids content of about 50 % at a temperature of 115–120° F. before drying in a Kestner centrifugal spray-drier.

Sampling

(*a*) *Milk.* During the preparation of each powder the following samples were taken: raw milk; milk at 110° F.; milk after passage through the A.P.V. Heat Exchanger and after evaporation. Table 1 gives the analysis of these samples. The results show that the powders were made from milks fairly similar in composition initially and at each stage of manufacture. The figures for the partition of nitrogen show that most of the heat-sensitive lactalbumin and lactoglobulin was denatured by all the pre-heating treatments, and that raising the pre-heating temperature from 190 to 230° F. apparently caused no further denaturation. The fact that no gross decomposition of protein was caused by the

Table 1. *Composition and partial nitrogen partition of the raw milk, pre-heated milk and evaporated milk from which the powders were made*

	Powder no. 1 (190° F.)			Powder no. 2 (230° F.)			Powder no. 3 (230° F. regn.)		
Constituents	Raw milk	Pre-heated milk (190° F.)	Evaporated milk	Raw milk	Pre-heated milk (230° F.)	Evaporated milk	Raw milk	Pre-heated milk (230° F.)	Evaporated milk
Total solids	11·9	12·1	48·5	12·1	12·1	50·7	11·8	11·9	49·3
Fat	3·4	3·6	14·2	3·7	3·8	14·4	3·3	3·5	14·1
Crude protein (N×6.38)	3·0	3·1	13·2	3·0	3·0	12·3	2·9	2·9	11·7
Lactose (anhydrous)	4·5	4·5	18·2	4·7	4·6	19·6	4·6	4·6	18·9
	Nitrogen content of the fraction as a percentage of the total nitrogen								
Nitrogen fraction									
Casein	77·9	89·0*	89·4*	78·4	90·9*	88·0*	78·6	88·8*	85·6*
Non-protein (N.P.N.)	4·0	3·8	3·9	3·8	3·6	3·8	4·3	4·3	4·6

* Includes denatured lactalbumin and lactoglobulin.

pre-heating treatment is also indicated by the similarity of the non-protein values at each stage of manufacture.

Table 2 gives the results of a nitroprusside test (4) for sulphydryl groups in the raw and heated milks. This was only a qualitative test, and heating at 230° F. apparently produced little more than at 190° F. It was shown by the method of Townley & Gould (5) that free hydrogen sulphide was present in milk heated at both 190 and 230° F. Mercaptans were apparently absent, although this may have been the result of the instability of the mercaptans under the conditions of the test (6,7).

Table 2. *Nitroprusside test for sulphydryl groups in the milk at stages in the manufacture of the powder*

		Milk sample				
Powder	Pre-heating treatment	Raw	Pre-heated at 110° F.	Pre-heated at 190° F.	Pre-heated at 230° F.	Evaporated
1	190° F.	−ve	−ve	+ve	—	+ve
2	230° F.	−ve	−ve	—	+ve	++ve
3	230° F. (regenerated)	−ve	−ve	—	++ve	+ve

In Table 3 the results of the bacteriological examination of samples of the milks taken at successive processing stages are given. The samples were incubated at 32 and 37° C., but there was very little difference between the counts and only the figures at 32° C. are given. As would be expected, heat treatment of the milks markedly reduced the bacterial population, particularly the 230° F. treatments.

Table 3. *Geometric mean counts of bacteria at 32° C. per ml. of the liquid milk samples and per g. of the powders*

	Milk pre-heated to 190° F.	Milk pre-heated to 230° F.	Milk pre-heated to 230° F. with regeneration
Bulk raw milk	938,700 (890,000–990,000)	1,244,000 (1,060,000–1,460,000)	830,500 (570,000–1,210,000)
Milk in balance tank	1,195,000 (930,000–1,460,000)	838,300 (398,000–2,110,000)	972,500 (850,000–1,160,000)
Milk from A.P.V. Heater	947 (490–1,570)	5 (1–10)	6 (3–12)
Condensed milk from evaporator	3,330 (320–9400)	34 (10–90)	39 (20–40)
Condensed milk from atomizer feed tank	5,350 (1,100–6,700)	229 (100–800)	1,010 (200–3,900)
Powder	4,130 (2,200–31,000)	1,562 (100–30,000)	2,970 (1,000–8,100)

Remarks. The figures in parentheses give the range of colony counts.

(*b*) *Powder.* After each powder had been running from the drying chamber for 30 min. it was sampled continuously throughout a 2000 gal. 'run'. Powder was not collected from the filter bags. Five 21 lb. cans of each powder were filled over a period of 3–4 hr. The five cans of each powder were emptied into a large bin, thoroughly mixed to ensure homogeneity, and repacked into the 21 lb. cans. These were then sent to the Hannah Institute for repacking into 8 oz. cans for storage. The composition of the three powders was very similar, the average figures being: moisture 1·9%, fat 28·7%, crude protein (N × 6·38) 25·6%, lactose (by difference) 37·9%, ash 5·9%; the maximum difference between the percentages of any constituents in the three powders was 0·2. Table 4 shows

the detailed partition of the nitrogen (8) in the powders and the milks from which they were made. The figure for albumin plus globulin recorded for the raw milk was not determined by direct analysis but is the value for albumin plus globulin in the powder together with the difference between the casein values of the powder and the raw milk. The globulin contents of the three powders, determined by Rowland's method (9), were 0·2, 0·1 and 0·0% respectively of the total nitrogen. It will be seen that most of the albumin-globulin fraction of the raw milk was denatured and to an equal degree by all three heat treatments. The values for proteose-peptone are the difference between 100 and the sum of all the other constituents, and hence include all experimental errors.

Table 4. *Partition of nitrogen in the milk powders compared with the corresponding values for the raw milk from which the powders were prepared*

	Percentage of total nitrogen					
	Powder no. 1 (190° F.)		Powder no. 2 (230° F.)		Powder no. 3 (230° F. regenerated)	
	Powder	Raw milk	Powder	Raw milk	Powder	Raw milk
Casein N	89·3*	77·9	90·3*	78·4	89·5*	78·6
Lactalbumin plus lactoglobulin N	1·6	13·0	1·5	13·4	1·9	12·8
Proteose-peptone N	4·0	2·8	3·1	2·2	3·3	1·8
Non-protein N	5·1	6·3	5·1	6·0	5·3	6·8

* Includes lactalbumin and lactoglobulin denatured by heat and thus precipitated with casein during analysis.

Table 5 gives the solubility of the fresh powders. The method of measuring solubility was that of Parsons (10), with the slight modifications of a powder to water ratio of 1 to 7, shaking for 30 sec. and centrifuging at $1000 \times g$. All the powders gave a slight positive sulphydryl test. The copper content was determined by the method of Hetrick & Tracy (11), except that amyl alcohol was used in place of carbon tetrachloride. The copper contents were 1·2, 0·9 and 1·1 p.p.m., a similarity which eliminated any anomalies in keeping quality from this source.

Table 5. *Solubility of the freshly prepared powders*

	Solubility of the milk solids (%)	
Powder	At 20° C.	At 50° C.
No. 1 (190° F.)	95·5	100·6
No. 2 (230° F.)	97·4	100·5
No. 3 (230° F.) regenerated)	96·9	100·0

The bacterial counts of the three powders are given in Table 3 and show no significant differences from each other.

Storage

The powders were canned within 5 days of manufacture in unlacquered tinplate cans (8 oz. size), the outer seams of which had been given two coats of bitumen. The packing density was the same for all powders at 0·509 g./ml., and, if an apparent density of 1·335 g./ml. is assumed for the powders (12), the free oxygen content of the cans was 0·347 mg./g. powder.

Cans were stored at 17, 37 and 47° C. and removed at intervals for examination. The samples used in the biological tests were subsequently stored at −20° C. until required.

Analytical tests during storage

The chief criterion of keeping quality of a whole-milk powder is its taste, and this was assessed independently by two panels of tasters, one at the Hannah Institute and the other at Messrs Aplin & Barrett Ltd., Yeovil. The tests were made as already described by Henry, Kon, Lea & White (13) (in the present experiment a powder to water ratio of 1 to 7 was used). As a chemical check on the progress of fat oxidation the peroxide values of the powders were determined using the ferric thiocyanate method of Hills & Thiel (14) as modified by Chapman & Mackay (15). This method affords useful comparative although not absolute values.

A further physico-chemical indication of the deterioration of whole-milk powder is the progressive removal of oxygen from the air within the can, and gas analyses were done at each sampling.

Biological tests

The biological value and true digestibility of the proteins of the three powders were determined on young rats by the method of Mitchell (16,17) at an 8% level of protein intake (N × 6·38). Tests were made on the freshly prepared samples and repeated on the same samples after 6 months' storage at 37° C.

The diets had the following percentage composition: dried whole milk 30·0, salts (18) 4·0, margarine fat 1·5, sugar 12·0, potato starch 10·0, rice starch 42·5. The percentage of nitrogen in the diets was: diet with powder no. 1 (190° F.), 1·216; diet with powder no. 2 (230° F.), 1·236; diet with powder no. 3 (230° F. regenerated), 1·239. Details of the experimental and feeding technique have been described in full by Henry, Kon & Watson (19).

Baking tests

A comparison of the three powders with fresh milk as additions in bread-making was very kindly carried out by Miss R. Bennett of the British Baking Industries Research Association.

The three powders were incorporated in a bread-making recipe at levels of 2 and 6% of the weight of flour; fresh milk in equivalent quantity was also used, and all loaves compared with a control using water, flour, yeast and salt only.

RESULTS

Palatability

The average of the 'off'-flavour marks awarded by the tasters for each powder at each test was plotted against the time of storage to give the curves shown in Fig. 1. This diagram shows that at each storage temperature, the palatability of the three powders could not be differentiated and that they were therefore equal in keeping quality. This result was arrived at independently by both tasting teams. The time taken by a powder to reach 'off'-flavour score 1·0 is regarded as its effective storage life at the particular temperature of storage. On this basis, the storage life of the powders at ordinary temperatures was 330 days. At 37 and 47° C. the effective storage life was 165 and 75 days respectively. An increase in storage temperature of 10° C. thus increased the rate of deterioration of palatability by a factor of 2·2 between 37 and 47° C., but by a factor of

only 1·4 between 17 and 37° C. The temperature coefficient found in the earlier storage experiments with spray-dried whole milk (1,2,3), was approximately 2.

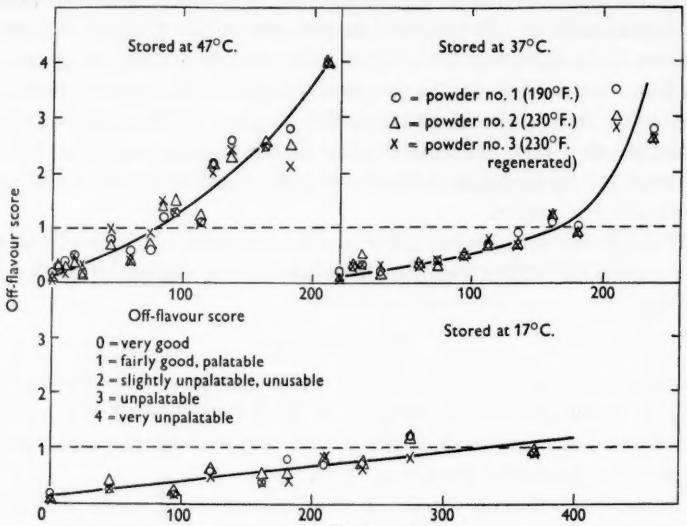

Fig. 1. The change in flavour of the three powders during storage at 17, 37 and 47° C.

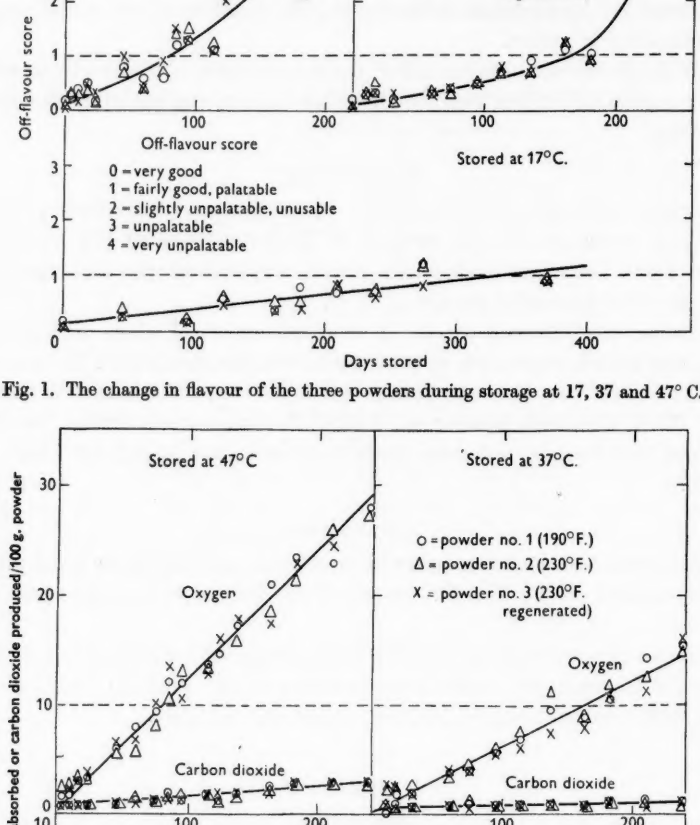
Stored at 47°C
Stored at 37°C.
○ = powder no. 1 (190°F.)
Δ = powder no. 2 (230°F.)
X = powder no. 3 (230°F. regenerated)
Oxygen
Carbon dioxide
Stored at 17°C.
mg. oxygen absorbed or carbon dioxide produced/100 g. powder
Days stored

Fig. 2. The absorption of oxygen and evolution of carbon dioxide by the three powders during storage at 17, 37 and 47° C.

At the initial tasting, the palatability of the fresh powders was considered to be very good but with a slightly 'cooked' flavour. The 'cooked' flavour was very slightly stronger in powder no. 1 (190° F.) and its initial 'off'-flavour score was 0·2, whereas powders no. 2 (230° F.) and no. 3 (230° F. regenerated) were scored as 0·1.

Absorption of oxygen and production of carbon dioxide

Fig. 2 shows the rate of absorption of oxygen and production of carbon dioxide by the three powders at the three storage temperatures. Each powder absorbed oxygen and produced carbon dioxide at approximately the same rate, depending on the storage temperature. Since the rate of absorption of oxygen is a sensitive indication of the rate of fat oxidation provided the moisture content is low (13), it is evident that the fat in each powder was equally resistant to oxidation and was oxidized at much the same rate. The temperature coefficient for absorption of oxygen was approximately 2·0 for an increase in storage temperature of 10° C.

Apparent peroxide values

It was possible to determine only the peroxide values of the powders stored at 47° C. immediately after opening the cans and these only up to the 75th day. The peroxide values of all three powders at this temperature were very similar, and on the 75th day were 1·8, 1·6 and 1·3 m.equiv./kg. fat for powders 1, 2 and 3 respectively; it is probable that at this time the oxidation of the fat was about at the end of the induction period. After each examination all powders were transferred to bottles and stored at 4° C. for peroxide determination when time permitted. The peroxide values of samples from each storage temperature were examined after 6–8 months, and although they were not then representative of the freshly opened material they showed very similar peroxide values for all three powders.

Biological tests

The results of the two tests are given in Table 6. They show that neither the biological value nor the true digestibility of the milk-powder proteins was affected by pre-heating at 230° F. instead of the usual 190° F., and that no change occurred in any of the samples after storage for 6 months at 37° C.

Table 6. *Biological value and true digestibility of the proteins of the dried milks when fresh and after storage for* 6 *months at* 37° *C.*

(Mean values with their standard errors for groups of 12 rats)

	Freshly prepared		Stored for 6 months at 37° C.	
Powder	Biological value	True digestibility	Biological value	True digestibility
No. 1 (190° F.)	87·7±1·3	91·8±0·8	87·1±1·7	91·6±1·0
No. 2 (230° F.)	88·7±1·4	90·4±0·7	88·4±1·0	92·2±0·8
No. 3 (230° F. regenerated)	88·3±1·2	91·1±0·8	88·9±1·6	91·0±1·5

Previous work (20) has shown that the biological value and true digestibility of the proteins of dried whole milk are not affected in any way when the pre-heating temperature of the milk is raised from 160 to 190° F. The latter temperature has also been shown (1) to cause no greater loss of vitamin A, carotenoids and vitamin C than the lower temperature.

Baking tests

At both levels (2 and 6%) fresh milk and the milk powders increased the volume of the loaf and improved the quality of the crumb compared with the control, the effect being much more marked at the 6% level. There was no significant difference in the bread-making properties of the three powders, nor was there any appreciable difference between the loaves containing equivalent amounts of fresh or dried milk.

DISCUSSION

The results of the keeping quality, biological and baking tests show that raising the pre-heating temperature of the milk from 190 to 230° F. did not improve the dried milk. The preliminary tests on the milks at the various stages of manufacture and also on the fresh powders had suggested that this conclusion was likely. This finding supports the conclusions of Findlay, Higginbottom, Smith & Lea (2), who used pre-heating temperatures ranging from 160 to 100° F., that no advantage was to be gained by raising pre-heating temperatures above 190° F.

The powder manufactured from milk pre-heated at 190° F. had a slightly more cooked flavour than the other two. This was somewhat unexpected but may have been the result of the extra time spent by the 190° F. milk in the balance tank prior to evaporation. The absence of greater quantities of sulphydryl compounds in the milk pre-heated at 230° F. may have been the result of volatilization during violent ebullition as these milks passed directly to the vacuum evaporator from the heat exchanger.

Since the sulphur-containing substances originate from protein breakdown it seems probable that the easily heat-denatured proteins, albumin and globulin, are the chief source. Both are rich in cystine, and if the heat treatment conditions favoured reduction, conversion to cysteine, mercaptans or free H_2S could easily follow. The sulphur compounds in themselves most probably function as antioxidants, as postulated by György, Stiller & Williamson (21) and Williamson (22), and may also contribute to fat preservation by reacting with the prooxidant copper present naturally or as a contaminant in milk.

The amount of amino-acid breakdown represented by the liberation of sulphur in the milks heated at either 190 or 230° F. had no deleterious effect on the nutritive value of the powders, the values shown in Table 6 being very similar to those found by Henry & Kon (20) for powder prepared from milk heated only to 160° F.

SUMMARY

1. Three samples of spray-dried whole milk were made from milk pre-heated at 190, 230° and 230° F. with regenerative cooling to 190° F., in an A.P.V. H.M. Paraflow Heat Exchanger and dried in a Kestner plant. The powders were packed in plain tin-plate cans and stored at 17, 37 and 47° C. The keeping quality of the powders was determined by examination at intervals for palatability, absorption of oxygen and peroxide value of the fat. The biological value and true digestibility of the protein of the powders were estimated in rat-feeding experiments initially and after 6 months' storage. The bread-making properties of the powders were also examined.

2. The keeping quality, biological and baking tests showed that the three milk powders were very much alike, and that no advantage was gained by raising the pre-heating temperature of the liquid milk from 190 to 230° F.

This experiment was made possible by the development of the H.M. Paraflow Heat Exchanger capable of running at 230° F. by Messrs A.P.V. Co. Ltd., London. This machine was installed by Messrs A.P.V. Co. Ltd. in one of the dairies of Messrs Aplin & Barrett Ltd., Yeovil, and to both these Companies the authors' thanks are due. The authors also wish to thank Mr S. A. Wilcox of Messrs Aplin & Barrett Co. Ltd., Frome, for producing the powders at Staplemead Dairy, and Miss Ruth Bennett of the British

Baking Industries Research Association for testing the baking qualities of the powders in bread. Dr C. H. Lea, Cambridge, Dr S. K. Kon and Mr A. Rowlands, Reading, and Mr C. H. Brissenden, Messrs A.P.V. Co. Ltd., took part in the original planning of the experiment.

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492. THE IMPORTANCE OF PSYCHOPHYSICAL ERRORS IN SUBJECTIVE JUDGEMENTS OF FIRMNESS OF CHESHIRE CHEESE

By D. SHEPPARD

National Institute for Research in Dairying, University of Reading

(With 1 Figure)

An experiment lasting 2½ months was carried out during 1949, in which some 200 cheese were examined using a large 'battery' of mechanical tests, while, in addition, subjective assessments were made for all the main quality ratings. The main results from these data have been reported elsewhere (1).

Between five and eight cheese were rated on each day by a group of three individuals, the same cheese being rated at 2 weeks and at 2 months old. Ratings for firmness and springiness were obtained through the rind; ratings for firmness, springiness, crumbliness and quality were made on a boring from each cheese.

One of the judges was a retired professional cheese-grader, while the other two judges were working on the mechanical testing of the cheese; nevertheless, one had spent several years working intermittently in cheese factories and had had considerable experience in judging the different qualities of cheese.

Harper & Baron have already published some interesting data obtained by studying these subjective judgements. One paper (2), however, is based mainly on the judgements made on the cheese at 2 months old, the judgements being those made in the main programme; the second paper (3) is concerned with sets of four or nine replicate judgements, on certain selected cheese, carried out in the intervals between the other experiments. The latter experiments have the disadvantage that the judges realized they were being tested for consistency, and were thus not judging under normal conditions.

Moreover, it is well known that any test in which replicate judgements are made on a set of materials may lead to their being recognized when re-presented, with the danger that the previous values allotted to them may be remembered. When four, or nine, replicate judgements are made, as in this case, unless the total number of cheese judged is very great by comparison, the test will be of little value as a test of judging skill.

The present paper is based on the assumption that an instrument called the 'ball compressor',* which was used throughout these tests, and whose readings gave a correlation of +0·86 with the ratings for the subjective concept of firmness, obtained by thumbing the cheese, can be considered as an 'objective measure' of firmness. This is, admittedly, only an assumption, but the mechanical instrument does closely imitate the subjective procedure, and it seems likely that if the psychophysical errors, as described below, could have been eliminated, the correlation between the results derived from the two methods of measurement would have been very high indeed.

The data discussed here were those obtained with the cheese when 2 weeks old, and were obtained on a total of 150 cheese.

* A machine by which a steel ball is driven into the cheese under a constant load. The readings are a measure of the indentation of this ball after a given period (30 sec.).

THE PSYCHOPHYSICAL ERRORS

Three of the main psychophysical errors were expected to prove important in these subjective assessments, and the data were analysed so as to extract their effects.

The first and most important source of error was that which will be described as an adaptation effect. This has been fully described by Tresselt and others (4,5,6), who have carried out a series of experiments to investigate it. In brief, it has been found that if there is any progressive change in a given quality of a set of materials, those which are judged as being of average quality will tend to change in value in the same direction. Furthermore, it is found that this occurs despite the previous experience of the judge in dealing with similar materials. Such an effect may be a genuine adaptation to a gradual change in the series, of a kind similar to physiological adaptation, resulting in the changes being noticed less than they should; or it may be a conscious effect, in which the judge thinks that the apparent change may be partly due to his becoming more critical, and compensates for this in his judgements.

The second psychological error is that described as the 'contrast effect', which has been investigated by Turner (7) and by Long (8). This becomes most important when there are considerable variations in the qualities of the different materials to be judged, and its effect is such that a higher quality rating will be given to a material when it is preceded by one of poor quality, than when it is preceded by a material of good quality.

A third effect, which has been described by Cohen (9), is for judges to tend to give judgements approximating to a 'normal frequency distribution' around their own mean judgement value. In cases where the distribution of the genuine objective values is not of this form, such a bias may be of considerable importance.

There are certain other important psychophysical errors which have not been mentioned, but this is because it has not proved possible to extract them from these data, rather than because they are not likely to be present.

ADAPTATION TO THE RANGE OF STIMULI

When judging for firmness the judges were asked to give a value for this quality on a points scale of 1–7, 1 point being given for 'very firm', 4 for a cheese of average firmness, and 7 for a very soft cheese. Each judgement was based on several thumbings of the cheese, and the corresponding ball-compressor readings were averaged from four readings for each cheese.

An examination of the ball-compressor readings showed that, over a period of some 3 weeks, in the middle of the experiment on the 2 weeks old cheese, the cheese being judged tended to become progressively firmer. It was also obvious that, although the judges were rating the cheese as becoming firmer, they were also giving the same firmness rating value to cheese which were considerably firmer at the end of this $1\frac{1}{2}$ months' period than to those to which they allotted this value at the outset. This effect was such that the grader was led to give the same value for firmness (2·0) to what were in fact the softest and the firmest cheese in the whole experiment.

It seemed, therefore, as if with every shift in the firmness range, the subjective values given tended to shift in the same direction.

To show this, a table was drawn up similar to that included in this paper, setting out objective firmness against serial order.

The serial order of presentation was divided up into groups of fives, since it seems that the cheese were probably judged in such groups, and the average firmness value for each of these groups was inserted in each group column. (In the table in this paper the mean value is represented by two arrows crossing the column.) Then, in each column the subjective values given to the cheese occurring in each group were inserted in the rows equivalent to their average ball-compressor values. A high objective value means that the cheese concerned was a soft one.

In the table shown here the presentation order was grouped into sets of fifteen judgements, since the full table was too large for publication, likewise the ratings for the three judges were grouped and expressed as average figures. As a result of this, some of the changes in the subjective values are masked by this grouping, while certain irregularities in the values shown within the columns themselves also occur as a consequence of this grouping. Nevertheless, most of the conclusions drawn could be arrived at by inspection of the table shown in this paper.

Table to show how the subjective assessments for firmness change in relation to the firmness of the series being judged

		Group									
Av. B.C. reading for groups ...		I 70·2	II 66·4	III 53·4	IV 57·6	V 51·4	VI 53·8	VII 54·8	VIII 42·0	IX 56·9	X 56·6
B.C. readings											
↑ Softness	77 – 79	4·1	4·0	—	—	—	—	—	—	—	—
	74 – 76	3·8	3·5	—	—	—	—	—	—	—	—
	71 – 73	3·6	3·1	—	—	—	—	—	—	—	—
		→ ←									
	68 – 70	3·1	2·7	—	—	—	—	—	—	—	—
	65 – 67	2·8	→ ←	—	—	—	—	2·8	—	4·2	—
	62 – 64	3·3	2·8	—	2·6	—	—	3·2	—	4·3	—
	59 – 61	2·3	2·6	—	2·5	—	2·8	—	—	3·6	3·3
	56 – 58	—	1·9	1·9	→1·9←	2·3	2·4	2·6	—	→3·2←	→2·9←
								→ ←			
↓ Firmness	53 – 55	—	—	→1·8←	1·8	2·0	→2·2←	2·9	—	—	2·8
	50 – 52	—	—	1·4	1·2	→1·8←	2·0	2·6	—	2·6	—
	47 – 49	—	—	1·1	—	1·7	1·5	—	—	3·0	—
	44 – 46	—	—	—	—	—	—	1·8	1·9	2·3	—
	41 – 43	—	—	—	—	—	—	—	→1·7←	—	—
	38 – 40	—	—	—	—	—	—	—	1·8	—	—

An examination of the table shows clearly that the subjects were differentiating very successfully within the groups, and this is even more evident when the data are grouped in fives. Thus the judges are evidently efficient at judging relative firmness.

On the other hand, if one examines any objective value which occurs frequently across the table, it is clear that the subjective ratings given as equivalent to it vary considerably and that, on the whole, these values become higher (softer) as the serial order progresses. Since there is a general trend for the cheese to become firmer as the experiment proceeded, this suggests that the judges were becoming 'adapted' to this effect, as previously suggested.

On the few occasions when the cheese tended to become softer for a short period, there were no significant changes in the subjective assessments. This is not clearly seen in the table given here, since many such changes are masked or enhanced by grouping the assessments into sets of fifteen.

A direct comparison from column to column shows even more clearly how the ratings can vary for the same objective values. For the first three columns the subjective ratings are reasonable and, were they grouped, a progressive decrease in the subjective values would appear down the columns. (One cheese, with an objective value of 47 ball-compressor units in group III, was assessed at a value of 1·0 by all three judges.) It is after these first three groups that the judges' mental scales seem to have become confused.

Thus in column IV a value of 1·2 is given for a cheese of the same objective value as one which had been judged previously as 1·4. In column V an opposite trend appears, and cheese judged previously as 1·9 and 1·1 are now given values of 2·3 and 1·7. Such effects appear in all columns from then on, and are particularly evident in column VIII, where cheese which are considerably firmer than the cheese in column III, which was judged as 1·0, are here judged as being 1·9 and 1·8.

In general, it can be seen that these changes in the subjective values occur when the average of the group changes, and that they are as previously described. A given cheese will be judged as softer when the group in general is becoming firmer, though the reverse effect does not always occur. These effects are even more apparent when the data are grouped in fives.

An examination of the data in the full table shows that the grader's judgements are a function almost entirely of the series being presented. The semi-expert was less affected by the changes in firmness value of the groups than was the grader. The non-expert, however, managed to maintain her mental scale at a very constant level throughout the test, and was only really confused by the sudden increase in firmness occurring in group VIII, the effect of which could be seen in the judgements on that group and in the two groups following. In fact, all the judges were more affected by the sudden change in firmness values occurring in group VIII, than by the gradual changes occurring previously.

There is one more result of this adaptation, which can be seen when one tries to construct a subjective scale for firmness in terms of the objective measurements equivalent to these assessments.

Such a subjective scale is shown in the figure given with this paper, where the averaged ball-compressor values, corresponding to each subjective rating, are plotted against these subjective ratings. Thus, for example, all the ball-compressor readings for cheese which have been given an average subjective rating of say 1·3 by one judge are averaged, and this average ball-compressor value plotted against the subjective value of 1·3 in the figure. The number of cheese for which any one subjective value was given is indicated above each point in the figure.

From this, it can be seen that if a particular subjective rating is only given when the cheese are at one extreme for firmness, certain anomalies may appear. Thus, in the subjective scales shown in the figure, the grader's judgement of 1·7 for firmness, comes between his values of 2·0 and 2·3 when compared with the objective scale, although the former value is an average based on twenty-three judgements.

Such an effect might be very serious if one were attempting to build up a set of materials of different standards for making comparisons, having no objective measure of the quality concerned.

It should be noted that the points which fall best on the lines are those which are averages based on a large number of judgements. These judgements were usually

scattered in time throughout the experiment, hence it is the judgements which are less adequately determined that fall in unusual positions in the subjective scales. In the majority of cases, the points are high.

It is also interesting that the intervals between judgements on the subjective scales may be very unequal in terms of the objective scale. For example, the semi-expert's judgements of 3·0 and 3·5 are 6·0 ball-compressor units apart, while the interval between

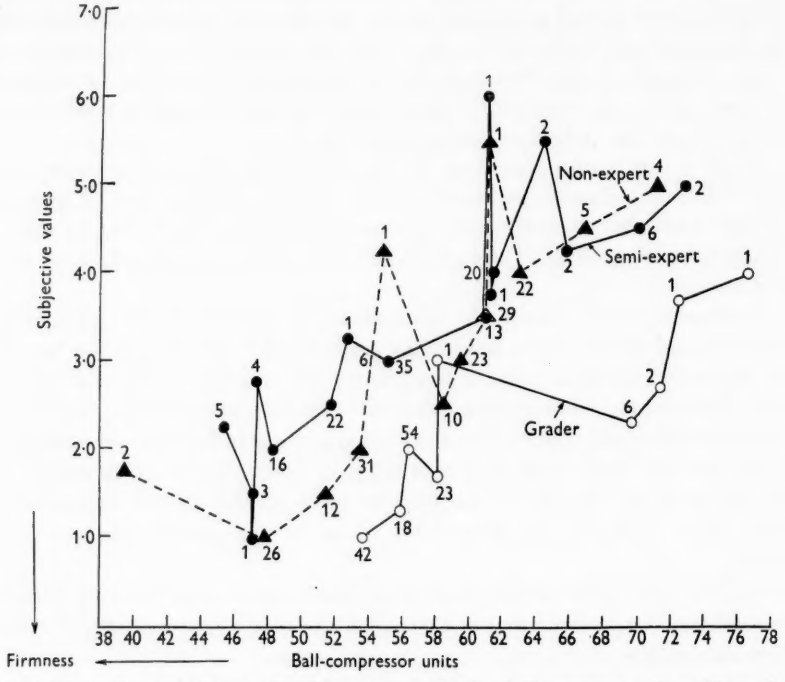

Fig. 1. Showing how subjective scales of firmness are related to ball-compressor values. Numerals refer to number of tests.

her judgements of 3·5 and 4·0 amounts to only 1·0 ball-compressor unit, although each one of these points is an average of twenty judgements or more. It is possible that this also is an irregularity caused by the changing value of the subjective judgements. It is also interesting to observe that in spite of the marked individual variations, the general relationship between subjective and objective values would seem to be fairly linear. It might have been expected, on the basis of the well-known Weber-Fechner laws, to have been a logarithmic relation.

CONTRAST EFFECTS

It was difficult to isolate these in view of the more important effect resulting from the trends in firmness of the cheese. Inspection showed that most of the points which deviated markedly from the general trend of the shifting subjective scale could have been so judged because of a contrast effect. In other words, such judgements were generally made on a cheese which was immediately preceded by a cheese of a softness or firmness unusual at that period.

It should be noted, however, that the cheese may not invariably have been judged in a serial order corresponding to the numbers allotted to them, since the order in which the judgements were made was not recorded at the time. The judges have stated, however, that they were usually judged in this order.

FREQUENCY DISTRIBUTION OF JUDGEMENTS

Grouping the frequencies of the objective measurements of firmness produced a curve slightly skewed towards the firm end of the scale, although it was not significantly different from a normal frequency curve calculated from the same data ($\chi^2 = 0{\cdot}50$).

A frequency curve for the semi-expert's judgements seemed to approximate more closely to a normal distribution curve than the objective values warranted ($\chi^2 = 0{\cdot}95$) although it is obvious that the difference between the two distributions would not be statistically significant.

The non-expert gave an artificially high number of 1·0 judgements, and it is probable that some of these would have been allotted to an even higher value had it been possible to do so on the rating scale used. Even taking this into account, however, the frequency of her judgements was significantly different from a normal frequency distribution.

However, since the objective values of the firmness of these cheese do approximate so closely to a normal frequency distribution, one cannot conclude from these data that the judges would tend to constrain their judgements into such a form even if the data had been differently distributed. In fact, except for the semi-expert, it seems improbable that they would have done so.

A further interesting point is that the semi-expert's intermediate judgements (e.g. 1·5), derived from an average of two separate ratings given for the same cheese, but which were of different values, exactly fitted her frequency distribution curve. The non-expert gave rather less 'intermediate' ratings, and the grader gave very few indeed. Thus the grader presumably very seldom changed his mind about 'borderline' samples, which suggests a bias due to his remembering his prior judgements, though this would be difficult to prove.

DISCUSSION

These effects raise some interesting points about subjective scales. Descriptive terms were included with the rating scale given to the judges, so that a judgement of 1·0 was to mean 'very firm', and a judgement of 7·0 to mean 'very soft'. Yet the grader and the non-expert gave more of their judgements at 2·0 (firm) than at any other value and the semi-expert gave the majority of hers at 3·0 (rather firm).

The grader, however, gave only six of his judgements greater than a value of 2·3, the semi-expert scarcely any greater than 5·0, and the non-expert gave few ratings greater than 4·5.

Does this mean that the judges really differ about the quality of firmness of the cheese 'due to a natural bias', as claimed by Harper & Baron, or does it mean something less obvious? There are always two ways of judging a series of variables, one is to compare them to an absolute standard, i.e. to say 'this is a very firm cheese'; the other is to judge the variables in terms of the series with which one is concerned, i.e. 'this is a very firm cheese for this batch'. In fact, most judgements probably depend on a combination of both these methods.

This suggests a different reason for these biases. Undoubtedly the grader had had a great deal of experience, and would be best able to judge that these cheese were, in fact, more than normally firm; the other two judges, with less experience, would be more likely to be influenced by the series presented to them.

Furthermore, the less expert graders were anxious to discriminate as widely as possible for experimental purposes, and would thus tend to give their judgements less frequently at the 'firm' end of the rating scale provided, and to give correspondingly more 'softer' judgements so as to make use of the whole scale. This effect can be seen clearly in the figure. Thus it may be that for these judgements the grader was not necessarily unable to discriminate widely, as previously considered(3), but may have been quite justified in bulking his judgements at one end of the scale. It is also likely that those with scientific training may have tended to make their judgements in terms of the code numbers given for the descriptions, rather than the descriptions themselves, which may also have helped to produce this effect.

A final point in this connexion is that with all the judges, their first five judgements were in the region of 4·0, the value for 'average firmness'. The objective data do not suggest that these were of 'average firmness', and it seems probable that the judges 'played safe' by giving them 'average' ratings, since they were not sure about their value in terms of the possible variations of Cheshire cheese. Then, having rated these, they went on to compare the cheese which followed with this original batch. Unfortunately for this method it happened that the first batch contained almost the softest cheese which they were to encounter.

CONCLUSION

Adaptation, contrast effects, and errors in the frequency distribution of the judgements have been traced, and their importance assessed.

Two problems arise from this. How can one trace such effects, and assess their importance, when there is no objective criterion? At present it seems as if a suitably planned self-consistency test, although not a good method, is the only possible one, and this has been fully described elsewhere(10, 11).

The second is to find some method of eliminating these effects. This is not easy to do, but it seems that when the most accurate judgements possible are required, the materials must be presented so that large differences between neighbouring stimuli, and continuous trends of certain qualities, do not occur; if necessary, and if possible, doing preliminary subjective or objective tests before carrying out the main series of judgements, so that the order of presentation can be carefully selected. No doubt one may be able to restrict the judges somewhat to making their judgements in terms of the general level of cheese qualities, or else in terms of the particular series being judged, by careful instruction, and by making sure that the judges have had a considerable amount of recent practice with the materials to which the comparisons have to be made. Although, as Harper and Baron have stressed, it is undesirable to disturb the natural method of judgement, it is obviously even more undesirable to have each judge making his comparisons on a different basis, if this can be avoided by giving them suitable instructions; nor are such instructions so disturbing as one might think, if they are followed by a practice period putting them into effect. Usually the judges will very rapidly become used to any slightly novel situation, provided it is not too different from their normal procedures, and provided that they are prepared to co-operate.

Finally, if one can arrange for the judges to have experience of really typical materials, and instruct them to make their judgements in terms of these, then it is obvious that the subjective assessments will be considerably more valuable. Furthermore, when instrumental tests have been standardized in terms of such subjective assessments, it will be easier to decide what value they will have for the industry.

Other experiments have just been completed (Sheppard, in process of publication) in which assessments of firmness were made with measurable stimuli on artificial materials, for which a similar adaptation effect was found.

SUMMARY

1. Ratings for firmness which were carried out on 150 Cheshire cheese at 2 weeks old, by three judges, were examined so as to extract the expected psychophysical errors.

2. It was found that the value for firmness given to any particular cheese tended to be a function of the average firmness of the group of cheese in which it was judged, so that a cheese of a given objective value would be given a different rating according to its position in the series being judged.

3. A cheese of a given value presented after a firm one is judged softer than if it had been presented after a soft one. This contrast effect was masked by the influence of the group as described in (2). Nevertheless, it seems probable that contrast effects influence the judgements.

4. The frequency distributions of the subjective judgements were examined, and it was noted that the 'semi-expert' judge gave subjective values which approximated very closely to a normal distribution, more nearly so than the judgements of the other two judges.

5. Reasons are suggested for the differences in the values allotted to the cheese as judged by the different judges.

6. Some suggestions are made for eliminating the effects described.

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REVIEWS OF THE PROGRESS OF DAIRY SCIENCE

SECTION E. DISEASES OF DAIRY CATTLE

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I. MASTITIS

A. *Eradication of* Streptococcus agalactiae

In a report of the Mastitis Committee of the Agricultural Research Council(1) details are given of trials carried out in various Research Institutes in Great Britain of intramammary infusions of penicillin in 50 ml. distilled water against *Streptococcus agalactiae*. In pilot trials four doses of 10,000 units at successive milkings sterilized 82% of the quarters infused, and eight to ten doses of 20,000 units at 24 hr. intervals sterilized 96% of the quarters. In the first conjoint trial carried out by various Institutes one dose of 100,000 units sterilized 58% of the quarters infused, whilst multiple doses of $4 \times 10{,}000$, $2 \times 20{,}000$ and $4 \times 20{,}000$ units sterilized from 80 to 83%. All quarters infused were positive for *Str. agalactiae* for three consecutive tests before treatment, and those which were taken as sterilized were negative for seven consecutive tests after treatment. In the second conjoint trial doses of $2 \times 20{,}000$, $2 \times 40{,}000$ and $2 \times 100{,}000$ units of penicillin sterilized 78–79% of the quarters infused.

That the treatment of all the quarters of all the cows in a herd, combined with disinfection of the animals and their environments, is the surest method of eradication, is shown by the work of Stableforth, Hulse, Wilson, Chodkowski & Stuart(2), who reported the eradication of *Str. agalactiae* from nine badly infected herds by simultaneou streatment of all cows with five doses of 100,000 units of penicillin at daily intervals accompanied by disinfection. Six herds were freed at once except for one cow which was freed later. Five of these herds remained free for 12–24 months. Some reduction in milk yields occurred on most of the farms during the week of treatment, but they increased in most herds after treatment. Clinical mastitis ceased to be a problem in all the nine herds. Penicillin in water was used in the early part of this experiment, but later it was suspended in arachis oil.

Though simultaneous treatment of all animals with penicillin appears to be the ideal method for eradication, the infusion of infected quarters only may also be successful. The amount of work involved and the time taken is, however, much greater. Edwards & Taylor(3) describe an attempt to clear a herd of 70–80 Ayrshire milking cows of *Str. agalactiae*. The herd was carefully managed, and even included the application of sulphanilamide to the milkers' hands. The udders were not stripped. Milk and teat swabs were examined by enrichment methods. For a period

* The years covered by this review were 1949–51.

before treatment the milk infection rate was steady at 15% of the teat swabs, but immediately before treatment fifty-four out of seventy-seven cows were positive by enrichment methods. Of these fifty-four cows, twenty-two were clinical cases and thirty-four had teat sores. All quarters were infused with 50 ml. of 30% sulphanilamide in an oil-water base on four successive days. There were six new infections during the period of the work, which meant that a total of sixty cows were treated. Two courses of treatment cleared infection from all but nine of these sixty cows (85% were freed). Penicillin was used to free the nine resistant cases and eight fresh infections. Only three further cases occurred from April 1947 to February 1948. Edwards & Taylor also report the clearing of *Str. agalactiae* infection from three out of four other herds. Francis(4) eliminated *Str. agalactiae* from five herds by intramammary treatment with 10–20 g. sulphone, and later by 10 g. sulphone plus 50,000 units of penicillin. As a rule cows were not treated till they were giving less than 2 gal. milk/day; and the infusion was left in the udder for 3 days. In dry cows it was left in the udder until the animal calved. Francis found that a single dose of sulphone and penicillin appeared to be as good in the lactating animal and better in dry animals than multiple doses of penicillin. The work of McFarlane & Rennie(5) confirmed that it was more satisfactory to treat dry cows or cows drying-off, in which the drug could be left in the udder, rather than actively lactating cows from which it is removed in the milk. They injected a suspension of vegetable black into udders, and found that in a gland milked out before the injection and not milked again before slaughter, the vegetable black was generally found to be present in the inflamed areas.

The importance of disinfection of the cow and its environment is shown by the work of Chodkowski(6). In 13 herds he recovered *Str. agalactiae* in 38% of the tests made on teats, in 5% of the tests made on skin and in 39% of the tests made on milkers' hands. The corresponding values for tests made on clothes were 20%, and for air, utensils, floors, etc., 22%. In two herds the organism was recovered from 61–81% of teats with sores and from 15–17% of teats without sores. Various materials artificially infected remained positive from 1 to 20 days. Sterilized materials when soaked in infected secretion and kept in closed tubes remained positive up to 2 months, whereas with unsterilized materials left open the time was only about 2 weeks. The same author(7) made trials with various disinfectants and found that C.T.A.B. (a quaternary ammonium compound and a synthetic cationic detergent, containing 75% of cetyl-trimethyl ammonium bromide), in concentrations of 1:500–1:1000 and also iodine solution (1:500–1:800) were the most effective against *Str. agalactiae* on the skin and in the environment of the cow. C.T.A.B. or formaldehyde gave the best results for the cowshed and fittings, C.T.A.B. for dairy utensils and C.T.A.B. plus penicillin cream for teat sores. Chodkowski & Lancaster(8) describe methods for the recovery of *Str. agalactiae* from outside the bovine udder. The method finally adopted for sites other than faeces, air and hands consisted of swabbing the area and then agitating the swab in a tube of sterilized milk containing potassium tellurite 1:20,000 and boric acid 1:500. After incubation for 2–3 hr. at 37° C., 0·1–0·3 ml. of the milk was spread on a sheep blood agar plate containing crystal violet 1:750,000, aesculin 1:1000 and thallium sulphate 1:3000. The results were read after incubation at 37° C. for 48 hr. The method used for the detection of *Str. agalactiae* in the air consisted of exposing milk in a Petri dish protected by a ¼ in. mesh wire gauze for 6–20 hr.

As Stableforth(9) points out there is no longer any doubt that mastitis caused by *Str. agalactiae* can be adequately controlled, and that the eradication of this infection from large numbers of herds is practicable. Treatment of all quarters of all cows in a herd yields excellent results; one-third to one-half of the herds so treated clearing at once and the remainder quickly. Infections other than *Str. agalactiae* can, however, be a cause of serious loss in some herds, and must become relatively more important as *Str. agalactiae* is brought under control. Further work is needed on the chemotherapy of these infections and the factors which favour their occurrence and development. In the meantime proper sanitary precautions and good milking methods remain essential to assist in their control. Francis(4) is of the opinion that eradication of *Str. agalactiae* from a herd, area or country may be simpler and less costly than the eradication of tuberculosis.

Reid(10), who compared five herds which were free from *Str. agalactiae* with five infected herds,

found little difference in the incidence of mastitis. He also found that a greater percentage of animals in the free herds were disposed of on account of udder trouble. However, the question whether the increase of other infections is real or only relative should not be allowed to interfere with any programme that may be devised for the eradication of *Str. agalactiae*.

B. *Treatment*

Though most of the work on treatment has been concerned with the infusion of antibiotics into the udder, the actions of the sulphonamides, either alone or combined with other drugs, have been studied. Carmichael, Maclay, Johnson & Johnson (11) report promising results in the treatment of penicillin-resistant mastitis caused by *Staphylococcus aureus* and *Streptococcus uberis* with a preparation containing 200 mg. dibromopropamidine and 50 mg. sulphathiazole in 5 ml. of an arachis oil-beeswax base. They claim as a rationale for this treatment that the propamidine is antagonized by the lecithin in milk, whereas sulphathiazole is potentiated by lecithin. They employed strict diagnostic methods, using direct plating for two pre-treatment tests and assuming that there had to be at least 100 coagulase-positive staphylococci per ml. before a sample was regarded as positive. For a test to be regarded as negative after treatment there had to be complete absence of the organism from two weekly samples when enrichment methods were used. Of forty infected quarters *Staphylococcus aureus* was eliminated from thirty-two by one or two courses of treatment. Six of these quarters relapsed but cleared up with a further course of treatment. Of six quarters infected with *Streptococcus uberis* five were cleared by this preparation. The preparation seemed to be effective against *Str. agalactiae* infections, but undesirable reactions to the injection were more marked and more frequent when this organism was present than with the others. Allen (12), using dibromopropamidine in latent udder infections with haemolytic staphylococci, found only temporary clearing of the infection, whereas with a mixture of this drug and sulphathiazole the infection was cleared, the milk yield increased and there was a reduction in streptococcal infections. During treatment, however, the milk was often greasy and unfit for bulking, though no systemic effects were noticed.

Simon, Spencer, Kraft & Schenk (13) compared the action of sulphone and penicillin separately and combined in the treatment of streptococcal mastitis. They found that intramammary infusions of sulphone and penicillin combined showed little evidence of being superior to penicillin alone. After treatment with sulphone in the form of Udolac, thickened milk was noted at the first, second and sometimes up to the fourth milking. The penicillin was suspended in Penicle, a commercial oil-water emulsion. Edwards (14) studied *in vitro* the mode of action of sulphanilamide and penicillin on *Str. agalactiae* in milk. He found the action of sulphanilamide to be different in fresh milk from that in milk heated to 90° C. for 10 min. In fresh milk the drug was bacteriostatic in concentrations of 2–200 mg./100 ml. Whereas in heated milk, after a lag period of from 7 to 12 hr. during which the organisms multiplied, the drug was progressively bactericidal. He found that the addition of *p*-aminobenzoic acid (P.A.B.A.) to the milk stopped the efficacy of the drug, and he considered that the delay in its action may be explained by the fact that the drug became effective only when the multiplying organism was unable to synthesize P.A.B.A. at a sufficient rate to oppose the competitive action of the sulphanilamide. The action of penicillin does not appear to be related to P.A.B.A. but to the phase activity of the organism. In fresh milk, where growth of the organisms is delayed for 6 hr., concentration of 0·06–1·0 units of the drug destroyed organisms in 24 hr., whereas in milk treated by heat for 10 min. they were destroyed in 6 hr. Spencer, Kraft, Simon & Schenk (15) compared the action of penicillin with that of other drugs combined with penicillin on animals with streptococcal mastitis in fifteen herds. They found that penicillin alone was as effective as penicillin plus sulphadiazine, or penicillin plus sulphamethazine, or penicillin plus bacitracin. There was no significant difference in the results obtained when 200 mg. of aureomycin were used as an ointment as compared with 100,000 units of penicillin in Penicle. Quarters selected in these experiments were usually positive for three examinations before treatment, and after treatment the number of streptococci in the milk was small. The Hotis test and microscopic examination of incubated samples were found to be unreliable after treatment, because they were negative in many samples which

were shown to contain streptococci by plating technique. The authors stress the danger of issuing enthusiastic research reports on the efficacy of drugs which may later cause disappointment to laymen, who have come to think that treatment will solve the mastitis problem instead of regarding it as being an aid to its control. In the treatment of staphylococcal infection Mundane(16) reports that a mixture of sulphone and penicillin was more effective than either alone. Fuller(17) reports that one to four infusions of 50 ml. of sulphamethazine plus 50,000 units of penicillin brought about clinical cures in 94·6% of sixty-four cows. The milk was stated to be fit for human consumption in 3–5 days. Berger & Francis(18) report on the use of sulphone alone or combined with penicillin in the treatment of mastitis. Buxton(19) describes two outbreaks of mastitis with *S. pyogenes* and *Str. agalactiae* co-existing. The former organism occurred in 25% of the cows infected in one herd and in 64% of the cows in the other. It was much more resistant to treatment with penicillin than *Str. agalactiae*. Schlingman & Manning(20), using penicillin in a bland ointment, report that an average of five doses was required to eliminate streptococci and staphylococci from twenty-two out of twenty-five quarters in eleven infected cows. Using the microscopic examination of incubated milk for diagnosis, Collinson(21) administered varying doses of staphylococcus toxoid to cows showing staphylococci. Quarters showing streptococci as well as staphylococci received intramammary infusions of penicillin in addition. All but one cow became clinically sound, but 20% of the quarters remained positive to microscopic examination.

Considerable attention has been paid to penicillin levels in the udder. Hughes, Christie & Farmer(22) compared the levels in the udder of procaine penicillin and calcium penicillin in arachis oil and beeswax. After infusing 100,000 units of procaine penicillin into eight lactating udders they found traces in two of them after 72 hr., whereas after infusing 100,000 units of calcium penicillin into eight udders no trace was found after this time. On the assumption that the descendants of penicillin-resistant organisms are susceptible, they tried interrupted treatment. Calcium penicillin given at intervals of 72 hr. cleared *Str. agalactiae* from all but one of twenty-five quarters, and procaine penicillin given at the same intervals cleared *Str. agalactiae* from all but two of twenty-one quarters. Foley, Stults, Lee & Byrne(23) studied different vehicles for sustaining penicillin levels in the udder. The use of mineral oil, water and lanoline derivatives as a vehicle was not so efficient as when a small quantity of propylene glycol and a non-ionic wetting agent were added. Bolton, Frayer, Cady & Waller(24) found that the infusions of 25,000 units of procaine penicillin G yielded as satisfactory milk levels after 8 hr. as when two to three times that amount was used. Trussell & Stevenson(25) reported on the use of bougies containing 25,000 units of amorphous calcium penicillin. In their view the residual milk in the quarter and the milk secreted between milkings acts as a solvent and vehicle for the distribution of penicillin in the gland, thus rendering unnecessary the use of other solvents. The penicillin concentration of the fore-milk and last milk was found to be more than adequate to maintain a therapeutic level. Jackson(26) compared the level of penicillin in the milk after injection of a variety of penicillin medications into cows at the end, middle and start of the lactation period. In general the highest levels were obtained at the end of lactation and the lowest at the beginning. Procaine penicillin in oil containing aluminium monostearate gave measurable levels for the longest time. When 300,000 units were administered per quarter a measurable level was obtained for 72 hr. at the beginning of lactation and for 192 hr. during the middle of lactation. When 25,000 units or more of any form of penicillin were administered to cows in all stages of lactation, measurable milk levels were obtained at the 24 hr. sampling period, and for longer periods during the latter part of lactation.

That penicillin sensitivity can be induced in some organisms by preliminary growth in the presence of a sensitive organism has been shown by George & Pandalai(27). For example, *Bacterium coli*, sensitive to 10 units of penicillin per ml. of medium, was inhibited by one unit per ml. after growth with *Staphylococcus aureus*. The staphylococci also became more sensitive. Tests carried out with fractions derived from penicillin-sensitive organisms indicated that the sensitizing factor was in the ribonucleic acid moiety. It appears that when penicillin-resistant organisms are grown in the presence of nucleic acid extracted from penicillin-sensitive organisms

the nucleic acid becomes part of the cellular material of the resistant organisms and renders them sensitive. Gale & Rodwell (28) suggest that the resistance to penicillin in some strains of *Staph. aureus* is linked with the organism's ability to synthesize its own amino-acids from ammonia and glucose.

Regarding the question of treatment with a combination of penicillin and streptomycin, some success has been claimed by Foley & Byrne (29). They used a mixture of streptomycin (0·1 g.) and penicillin (100,000 units) in 20 ml. of an oil-in-water vehicle (penicle) to treat a herd of forty-five lactating animals in which forty-five quarters were infected with *Streptococcus agalactiae* or *Staphylococcus aureus* or both or with *Streptococcus dysgalactiae*. Of twenty-four quarters which showed both streptococci and staphylococci, twenty-one were cleared with two treatments, and two quarters showing *Str. dysgalactiae* were cleared in one treatment. This mixture was used on the grounds of the dissimilarity of action between the two drugs, one being bactericidal (streptomycin), the other fissibactericidal (penicillin). Streptomycin injected with water was detectable for only 12–24 hr., but when injected in a water-and-oil vehicle it could be detected for 36–60 hr. Foley & Byrne used macroscopic and microscopic examination of the Hotis test for the diagnosis of streptococci, and for staphylococci microscopic examination was followed by a confirmatory plating test. This method is probably not stringent enough for post-treatment examination. Bougies containing streptomycin and penicillin were used by Bryan, La Branche & Drury (30). Bougies containing 50,000 units of each proved effective in the treatment of nine cases of acute coliform mastitis, one of staphylococcal and six of acute streptococcal mastitis. They were also successful in eighteen cases of chronic streptococcal mastitis. Cooper, Ford & Robinson (31) found that an ointment containing 50,000 units of penicillin and 50,000 units of streptomycin yielded 88–100% of 'cures' as compared with 0–100% when 'sulpha' drugs plus penicillin were used against *Str. agalactiae*, *Micrococcus aureus* and coliform infections. Fey (32) discusses the treatment of fourteen natural and two experimental cases of coliform mastitis with four infusions of 1 g. each of streptomycin in 100 ml. of saline during a period of 24 hr. Though affected quarters were sterilized, this alone did not influence greatly the cases with systemic involvement. Trussel & Stevenson (33) have studied the concentration of streptomycin in milk following the insertion of bougies into the teat. Assays of the fore-milk and strippings showed the presence of streptomycin 12 hr. after insertion. The concentration varied inversely with the amount of milk secreted, and large initial doses maintained significantly higher levels for longer periods than did small doses. They saw no evidence of irritation due to the streptomycin.

Aureomycin is described by Broschard, Dornbush, Gordon, Hutchings, Kohler, Knupka, Kushner, Lefemine & Pidacks (34) as a new antibiotic principle active against certain viruses and rickettsia and against both Gram-positive and Gram-negative micro-organisms isolated from the substrate of *Streptomyces aureofaciens*. It is named from the yellow colour of the parent actinomycete and the golden brown colour of the crystalline antibiotic. Its physical and chemical properties are listed. Reports indicate that this drug is to some extent effective against mastitis caused by staphylococci, *Streptococcus dysgalactiae* and *Str. uberis*. Hulse (35) treated ten cases of acute staphylococcal mastitis with two infusions of 400 mg. of aureomycin hydrochloride in 7·5 g. of ointment, the two infusions being separated by an interval of 48 hr. The clinical symptoms were similar in all ten cases; there was acute mastitis in one or more quarters and general systemic upset. Half of fourteen affected quarters were restored to nearly normal by aureomycin alone or in conjunction with penicillin, sulphamethazine or dibromopropamidine. McCulloch, Kiser & Migaki (36) used aureomycin against staphylococcal mastitis in cows, and state that 100 mg. destroyed infection in 50% of cases at 21 days; 150 and 200 mg. were about 61 and 86% effective respectively. Packer (37), treating chronic bovine mastitis with aureomycin, found that one infusion of 200 mg. caused twelve quarters which showed *Str. uberis* and *Str. dysgalactiae* to become negative, whilst two infusions cleared staphylococci from 68·5% of seventy infected quarters.

Schalm (38) states that in most instances the shedding of a minimal number of pathogens following therapy indicates that the infection has not been overcome, and that in subsequent

samples organisms will usually be found in pre-treatment numbers. On this basis he found that aureomycin in multiple doses from 200 to 800 mg. did not produce results against *Staphylococcus pyogenes* mammary infections that could be considered an improvement over infusions of penicillin. He notes, however, that this failure may have been due to the inclusion in the experiment of eighteen quarters that had previously failed to respond to other therapeutic agents. Slanetz & Allen (39) report good results from the infusion of 200 mg. of aureomycin into 230 quarters, but mainly against *Streptococcus agalactiae*. This organism was isolated from 223 of these quarters, *Str. uberis* from four and *Str. faecalis* from three. In 212 quarters (92%) the infection was eliminated. One infusion of 400 mg. of aureomycin into fifty-seven quarters (fifty-six with *Str. agalactiae* and one with *Str. uberis*) was successful in freeing fifty-four (95%) of these quarters. Twenty-two quarters which had a chronic infection over a period of 2 years, twenty-one with *Str. agalactiae* and one with *Str. uberis*, and which had failed to respond to a single dose of 200 or 400 mg., were cleared of infection by three infusions of 200 mg. at intervals of 4 hr. Assays of the milk showed a high inhibitory concentration of aureomycin for at least 48 hr., even when the cow was milked at 12 hr. intervals.

Reports on the use of nisin, the antibiotic from *Str. lactis* are promising. Hirsch & Mattick (40) have described further tests with a purified form of the substance. It was found to be rapidly bactericidal for *Str. agalactiae* both *in vitro* and in mice. Toxicity tests on rabbits showed considerable variation with the route of administration. The lower toxicity by the subcutaneous route appeared to be due to very slow absorption, and this was confirmed by the fact that lower concentrations were obtained for longer periods by this route. Hirsch (41) describes two methods for the assay of nisin, a bactericidal method and a lag-phase method. In preliminary trials in the treatment of bovine streptococcal mastitis Taylor, Hirsch & Mattick (42) found nisin very irritant, but reduction of the particle size of the drug reduced the irritancy. Single doses of $2\frac{1}{2}$ million units freed thirty out of thirty-five cows from infection; and 5 million units freed thirty-six out of thirty-seven. Though tests *in vitro* against staphylococci were not encouraging, 5 million units were infused in ten cows yielding not less than 500 colonies per ml. Ten days later there was no infection, but at 21 days one cow was infected. In the above experiments animals selected for treatment were positive by direct methods at two tests made the week before treatment. Post-treatment tests were made at 10 and 21 days by direct and enrichment methods. Hulse & Lancaster (43) used nisin in chronic bovine mastitis caused by *Str. uberis* and by staphylococci. They found that the drug was detectable in the milk 48 hr. after a single infusion. The milk yield dropped and had not reached its previous level 13 days later. Two-and-a-half million units of nisin appeared to be successful in removing *Str. uberis* from seven out of ten cows where the infection was in one quarter. It did not appear to be successful in cows infected in more than one quarter. Haemolytic coagulase-positive staphylococci did not respond well to nisin.

Mires (44), basing his diagnosis on gross clinical criteria, such as the appearance of the milk and the bromthymol blue test, found that a single infusion of 25 ml. of 0·2% nitro-furazone was 78% successful in 7123 lactating cows and 90% successful in 3418 dry cows. In the dry cows the drug was left in the udder from 4 to 6 weeks before calving. Heishman (45) reports that though the antibiotics subtilin and bacitracin showed a significant synergism when used together, in clinical trials they did not prove superior to the other agents available.

Administration of drugs by routes other than by the way of the teat canal have been reported. Schalm, Bankowski, Ormsbee & Browne (46) tried the effect of sulphamethazine against mammary infections of *Staphylococcus aureus* and *Aerobacter aerogenes* by oral, intravenous and intramammary administration. In only a small percentage of animals were the organisms eliminated. Clinical improvement as shown by the strip cup was not evident, and there was an increase in the leucocyte count. Lower (47) found that intravenous administration of 5 mg. aureomycin/lb. body weight plus 200 mg. by intramammary infusion was highly successful against mastitis. Easterbrooks (48) described a case of haemolytic staphylococcal mastitis and generalized infection following a teat injury. It failed to respond to intramammary infusion of penicillin and aureomycin, but responded to a total of 27·5 g. aureomycin intravenously given over a period of 5 days. Acute infections of the udder caused by *Staphylococcus aureus* leading to severe systemic upset

are discussed by Turnbull (49). With good reason he puts the questions, 'Why must a therapeutic agent need to be diffusible into the milk before it is of use in mastitis? Would it not be more rational, in acute mastitis at any rate, to endeavour to achieve bacteriostatic or bactericidal levels in the capillary networks? To remove the milk and pus from the gland, rather than to medicate those contents *in situ*?' He claims success, except in advanced cases, by the injection of a million units of sodium penicillin in 5–10 ml. of boiled water subcutaneously plus 1·25 million units penicillin B.P. intramuscularly. He states that infection is usually through a wound in the skin of the gland or the teat. Morse (50) has studied the response of cattle to penicillin preparations following intramuscular injection.

C. *Summer mastitis*

Valuable work on summer mastitis has been reported during the period under review. Marked increases in blood antitoxin levels in cows suffering from mastitis associated with *Corynebacterium pyogenes* were demonstrated by Weitz (51). Cows with infection of the uterus showed no increase in antibody titre. He found no evidence of protection following the use of *C. pyogenes* alum-precipitated toxoids or commercial vaccines, and thinks that the antitoxin produced may not be the antibody concerned in protection. Lovell, Foggie & Pearson (52) carried out field trials in Ulster during the summer months of 1942–5 in order to assess the prophylactic value of *C. pyogenes* alum-precipitated toxoid against summer mastitis. Infection occurred in forty out of 2123 immunized cattle (1·9%), and in forty-seven out of a control series of 2122 cattle (2·3%). Though agreeing in part with the view of Weitz that the antitoxin produced may not be the antibody concerned in protection, these authors are of the opinion that, though mastitis is not prevented, mortality is reduced. They point out the need for a study of the general problem of antibody response to non-living vaccines in cattle. Pearson (53) reports preventive treatment in 3200 dry cows with calcium and procaine salts of penicillin in an oily base with and without aluminium monostearate in an attempt to reduce the incidence of summer mastitis. He estimated that this procedure resulted in 50–70% protection, and there was strong evidence that if the period on summer mastitis outbreaks could be forecast or if it coincided with the time of teat inoculations the degree of protection could be higher. He pointed out that in attempting to do further experiments, problems would arise in connexion with the prevention of control animals from being treated; farmers have access to penicillin and might use it on the controls. In addition to *C. pyogenes*, Stuart, Buntain & Langridge (54) isolated from the secretions of quarters affected with summer mastitis, an anaerobic coccus, a microaerophilic coccus and *Streptococcus dysgalactiae*. Infusion of a mixture of the four organisms into thirty-eight dry quarters, caused summer mastitis in thirty-three of them, and there was pus in the cisterns of the other five. Infusion of *Corynebacterium pyogenes* alone into twenty quarters caused summer mastitis in only four of them. The authors note that neither the anaerobic organism nor the micraerophilic organism have been reported as occurring in lactating udders, but they observed that this may be because anaerobic cultures are rarely made.

D. *Experimental mastitis*

Lancaster & Stuart (55) carried out experiments on the transmission of *Streptococcus agalactiae* infection by milking with infected hands. Milk infected with Type III*a* was smeared on the teats of eighteen cows immediately before milking for a period of 15 weeks. Twenty-seven quarters of twelve cows became infected, and by the twelfth to the fifteenth week the clinical and other effects resembled those of a severe outbreak of *Str. agalactiae* mastitis. Six quarters with a pure infection showed no clots in the milk. In seventeen quarters the average time which elapsed after infection before clots showed in the milk was 2–3 weeks. Stuart & Lancaster (56) found that quarters which were relatively more susceptible to infection with *Str. agalactiae* after milking with infected hands were more susceptible to re-infection by infusion of this organism by way of the teat canal. They consider that about 2000 organisms will infect a susceptible cow. Lancaster & Stuart (57) describe 114 attempts to infect thirty-one cows of varying age with *Str. agalactiae* over a period of three lactations, by infusion, by hand-milking and by way of the

milking machine. Some cows were very susceptible to repeated experimental infections, whilst others were exceedingly resistant. In most of the cows susceptibility increased with age or with previous infection. The experimental work on summer mastitis carried out by Stuart *et al.*(54) has already been referred to.

Using twenty strains of Group B streptococci of bovine origin Pattison(58) studied their pathogenicity for mice. There were considerable variations in pathogenicity when the organisms were grown on different media. Strain S13 on intraperitoneal inoculation caused a fatal bacteraemia. Pattison(59) also found that 1000 units Procaine G penicillin would protect mice against a test dose of strain S13, and that repetition of the test dose at intervals of 4–7 days created an active immunity.

In a series of five papers by Pattison and his colleagues studies on experimental streptococcal mastitis in goats are discussed. In the first of these papers Pattison, Taylor & Holman(60) describe work in which they found that inoculation of 100,000 million viable organisms of *Str. agalactiae* into the teat canal gave rise to a sharp systemic reaction that lasted 2–3 days. This was followed by streptococcal mastitis which was similar, clinically, biochemically and bacteriologically to bovine streptococcal mastitis. The same number of heat-killed organisms produced a brief systemic disturbance, slight abnormalities in the milk and slight transitory enlargement of the udder. The reactions to the inoculation of Seitz IK filtrate of whole broth culture were practically negative. In the second paper Taylor, Holman & Pattison(61) report that the type and intensity of the reaction to the inoculation of viable *Str. agalactiae* could not be correlated with the number of organisms used. They noted that of 258 isolations of organisms from the milk of experimentally infected goats forty-five were more lethal for mice after growth in the udder. This is further discussed in the third paper(62), where the authors give methods for preparing mouse-protecting sera from goats. In the fourth paper Pattison & Holman(63), counting only what appeared to be 'healthy' neutrophils, state that when a million cells/ml. of the milk were taken as the normal figure all the pre-inoculation counts and 17% of the post-inoculation counts were below this figure. Inoculation of heat-killed organisms into the teat canal caused a rise to almost a million neutrophils per ml., but the count fell to about 100,000 and remained at this figure for 11 weeks. With living organisms the count was from 1 to 10 millions/ml., with a consequent drop in the number of these cells in the blood. They are of the opinion that the experimental disease in goats does not differ from naturally occurring bovine streptococcal mastitis. In the fifth communication Pattison(64) describes the histological findings in experimental streptococcal mastitis in the goat. He notes the patchy nature of the disease, normal tissue being found to a greater or lesser extent in all cases. Two goats killed 9 days after inoculation of heat-killed organisms showed a slight migration of neutrophils into secreting acini. No abnormality was seen in an animal killed 45 days after inoculation. The minimal reaction to the inoculation of living organisms was a patchy migration of neutrophils into secreting acini and a rather more generalized increase of interacinar cellularity, the cells being macrophages and fibroblasts. After 45 days some of the larger ducts showed a slight periductal cellular reaction of the macrophage type. Streptococci were difficult to find. The author considers this minimal reaction to be of a non-specific type similar to that caused by dead organisms. He divided the changes in what he regarded as the marked reaction into those occurring in the lobules and those occurring in the ducts. Those occurring in the lobules arose from a migration of neutrophils into secreting acini followed by a heaping up and vacuolation of the acinar epithelium. These changes were progressive up to 4 days and then the affected lobules started to involute, a process which the author considered to be a defence reaction. The changes in the ducts consisted of subepithelial infiltration of macrophages and a few neutrophils followed by papillary outgrowths from the duct walls.

The work of Spencer & McNutt(65) can conveniently be discussed here as it is complementary to that of Pattison. They studied the pathological alterations in twenty-five bovine mammary glands. The chief lesion described was an exudative inflammation into the acini similar to that described by Pattison in the goat(64). In chronic streptococcal mastitis they found both chronic interstitial inflammation and acute focal infiltration of lactating acini. Since the acini in the foci

were dilated, the acinar epithelium was vacuolated and clots of exudate were occasionally observed in an occulating position, the authors considered that the acute focal infiltration was due to the blocking of a small ductule by fibrin clots. They found that induration, as observed clinically, was due to the fact that blocking of ducts by accumulated exudate caused swelling and was not due to fibrosis. From the fact that thirteen out of nineteen carrier quarters (i.e. quarters eliminating streptococci but clinically normal) showed microscopic lesions, they concluded that most cases of streptococcal infection are really streptococcal mastitis. The authors described the organization of fibrinous clots, and stated that they were attached to the wall of the duct only for part of their length, the remainder of the clot being covered by one or two layers of epithelium. It is possible that these are not organizing clots, but that they are similar to the papilliform projections described by Pattison (64) for the goat, especially so in view of their epithelial covering. However, it is inadvisable to be dogmatic about the nature of any formed structure seen in the lumen of a duct, unless serial sections of the whole area in question have been examined.

Foley & Byrne (66) infected one quarter of a cow experimentally by infusing 1 ml. of milk from an infected quarter of another cow. They estimated that 3200 chains of *Str. agalactiae* were infused. Active infection was established as shown by the bacterial population and the leucocyte counts. Figures from this quarter and from a naturally infected quarter of another cow showed that the streptococci multiplied very rapidly during the first 12 hr. after infection. 'Leucocytosis' followed, and there was a drop in the number of streptococci present. Thereafter a continuous growth of organisms occurred, depleted with each milking but increasing rapidly from the residue. Infusion of 1000 units of penicillin caused an apparent disappearance of the organisms in 24 hr., but they were found in large numbers 2 days later. Infusion of 100,000 units of penicillin sterilized the quarters and reduced the cell counts. Sellars (67) found that fifty-two strains of *Str. agalactiae* examined by him produced hyaluronidase. No relation could be demonstrated between this activity and the clinical severity of the disease from which each strain was derived.

E. *Diagnosis*

Martin (68) describes a method for the microscopic examination of the milk in which milk samples are incubated for 15–18 hr. in inverted tubes, after which a film is made by applying the stopper to a glass slide. Heever (69) advocates the combined use of the Hotis and microscopic tests in the diagnosis of mastitis. Wilson & Slavin (70) describe a direct culture method for the examination of milk samples, using a face-cream type of jar with an aluminium lid. Plain agar is used to cover the convexity at the bottom of the jar with a layer of blood agar above it. The jars are incubated to test sterility and also to dry them. The fore-milk is not discarded, a small quantity from each teat being milked on to the surface of the medium, after which the jar is rocked and the excess milk discarded. The screw cap which contains an absorbent pad is replaced and the jar inverted. On arrival at the laboratory the jars are put in an incubator for 48 hr. at 37° C. and the numbers and kinds of colonies recorded. The authors state that the number of samples which can be dealt with has been increased fourfold by the use of these jars. Olsen (71) found by plating 4183 milk samples from 1058 cows that the fourth and fifth streams of milk drawn from a teat were less subject to contamination than the preceding three. Bosgra (72) recommends the freeze-drying of bovine plasma in small quantities in order to have a fresh supply for the coagulase test. He adds that this test may be used to great advantage in the routine bacteriological examination of milk.

Kruse (73) gives an account of the use of the rapid abnormality meter to detect cases of mastitis in the field. Jones (74) assessed the difference between the conductivities of the milk from the four quarters of an udder by deducting the lowest value from the other three. He compared this test with the resazurin test, and noted that abnormal milk is not necessarily detected by either test. Anderson & MacLeod (75) studied the effect of a period of non-milking on the leucocyte count of the milk using six experimental animals and two controls. Milking was suspended in the experimental animals for 10 days, and after this period milk samples showed a 27-fold increase in the leucocyte counts and there was a decrease of 85% in the milk yield.

Chu(76) compared five indirect tests and the culture test with the pathological findings in the udder. He estimated that the chloride test showed what he regarded as an 87% agreement with the histopathological findings. The corresponding figure for the catalase test was 92%, for the Whiteside test 82%, for the bromthymol-blue test 44% and for the total cell count 100%, whereas the culture test carried out on both fresh and incubated milk and on the udder tissue was not capable of identifying more than 45% of the affected quarters. The five indirect tests when compared with the culture test were only from 45 to 50% accurate. Chu claimed that the cell count was 100% accurate in diagnosing mastitis, but it should be noted that to obtain this high degree of accuracy he had to assume that a total cell count greater than 20,000/ml. of milk indicated mastitis. His findings were based on the results obtained by examining eighty-seven mastitic quarters. McFarlane, Blackburn, Malcolm & Wilson(77) found that of forty quarters showing inflammatory lesions on histological examination the milk from thirty-six had cell counts of over 100,000 cells/ml. Of fourteen quarters which were histologically normal the milk from only two had cell counts of over 100,000 cells/ml. The cultures made from the milk agreed with the histological examination in only 56% of thirty-nine quarters; the cultures made from the tissue in 62%. It is evident that indirect tests, the cell content of the milk in particular, can be more accurate in the diagnosis of an inflammatory process in the udder than the culture test. Whether an inflammatory process in the udder, from which no organisms are recovered from the milk, is due to the causal organisms being confined to the tissue or to the fact that they are destroyed by the milk, or whether the process is of a non-specific nature, is not known. Until there is more information on this subject, the finding of specific organisms in the milk must of necessity continue to be one of the main means of diagnosing mastitis, but the cell count test in itself can be of great value.

F. *General*

Various reports have been published referring to incidence and susceptibility. Spencer & Kraft(78) found in twelve dairy herds examined for mastitis by clinical and bacteriological methods that the incidence of streptococcal udder infection increased with advancing age, but at different rates in different herds. The rate of increase was correlated with herd management procedures which affected the degree of exposure. They considered that it was the degree of exposure to infection that caused the increase of infection and not the age of the animal. Composite milk samples from 7751 cows were examined for streptococci by Alberts & Bryan(79). The incidence of streptococci increased from 23·9% in first lactation animals to 85·7% in those in their twelfth lactation. They estimated that seven out of every eight cows that entered the milking line were removed before the sixth lactation. Fincher(80) after examining 167 herds concluded that the incidence of mastitis was related to bad herd management. He considered that the incidence of mastitis can be reduced by clinical examination alone, and that(81) treatment should be considered as the last resort. Berger & Francis(82) estimated that mastitis due to *Str. agalactiae* occurred in 41% of the quarters examined in herds they visited in a 3-year period. The figures for other organisms were: staphylococci 4·7%, *Corynebacterium pyogenes* 8·3% and other streptococci 31%. They found that the incidence of mastitis was four times greater in the first month after calving than during the next 4 months. Ineson & Cunningham(83) found from milk examinations made on fourteen herds over a period of 7 years, that new infections with *Streptococcus agalactiae* occurred about twice as often in the months of August, September and October as at other times. They found no statistically significant difference in the occurrence of new infections in the first, second, third or fourth lactations. Classifying cows and their daughters in twenty-seven herds in New Zealand as susceptible or resistant to mastitis according to whether or not they developed clinical mastitis before reaching 8 years of age, Lush(84) found statistically that susceptibility to mastitis has a strong genetic background. Spencer & Augevine(85) found that cows infected with *Str. agalactiae* responded to an intradermal injection of streptococcal antigen with larger and more persistent swellings than did normal cows, and that previously infected animals showed a severe inflammatory response to the intramammary injection of antigen. This illustrated that hypersensitivity of the bovine mammary gland is probably a factor in the pathogenesis of streptococcal mastitis. In a study prompted by the

'age-factor' hypothesis Ormsbee & Schalm(86) found that in a second outbreak of streptococcal mastitis in a large herd, 22% of the 205 cows previously infected and cured were concerned. In addition, 20% of the 236 not previously infected were also involved. The average age of the former was '5·01 lactations' and of the latter '3·53 lactations'. They therefore concluded that age *per se* does not play an important part in the epizootiology of mastitis caused by *Str. agalactiae*. Extended exposure to meet randomly distributed and transitory predisposing circumstances is regarded as the major factor. They make the point that the animals in a herd are not usually subjected to equal exposure; for example, the heifers are often milked first, and the infected cows last. Rømer(87) gives the incidence of infection in 9916 cows as *Str. agalactiae* 32·6%, *Str. dysgalactiae* 1·9%, *Str. uberis* 3·1%, *Str. pyogenes* 0·4% and staphylococci 1·0%. Neave, Dodd & Henriques(88) found by regular examination of the secretion from the udder during the dry period that about 50% of the infection which occurred during this period persisted, and that about half of the infection which persisted gave rise to clinical cases. Meigs, Burkey, Sanders, Rogosa & Converse(89) studied the relationship of machine-milking to the incidence and severity of mastitis. In a herd of twenty-five cows they compared hand-milking and three types of machine-milking. In 'routine machine-milking' they applied the machine for 14 min. at a pressure equivalent to 16 in. of mercury. In 'severe machine-milking' the pressure was also 16 in. but the machine was applied for 20 min. In 'mild machine-milking' the pressure and time were reduced to 12 in. and 5 min. respectively. The incidence of mastitis and decrease in milk yield were generally related to the intensity of the pressure adopted and to the length of the milking time. There was an improvement in cows with mastitis when they were changed from 'routine' or 'severe' machine-milking to 'mild' machine-milking; a further improvement occurred when a change to hand-milking was made. Schalm & Ormsbee(90) isolated *Staphylococcus pyogenes* from 2493 of 17,367 milk samples (14·3%), taken from a large herd over a period of 4 years. Infection was eliminated by penicillin therapy in 20·3% of lactating cows and 52·8% of dry cows. They were only considered free if the organisms were not found for 3 months following treatment. Treatment and segregation of affected animals reduced the incidence for the first 2 years of the experiment, but in the third and fourth years more animals became infected than were cured. The authors considered that penicillin resistance plays only a minor role in the failure to cure staphylococcal mastitis, and that localization of the organisms within the tissue is a more tenable explanation.

Infection of the udder with paracolon bacteria is reported by Johnson, Bruner & Murphy(91). An organism closely related to the Ballerup subgroup was isolated from forty-four quarters of seventeen cows. Treatment with terramycin, aureomycin, neomycin and streptomycin was ineffective, but appeared to reduce the number of clinical cases. Wilson & Lancaster(92) describe instances of acute bovine mastitis occurring in two cows and caused by *Diplococcus pneumoniae*, complete atrophy of the affected quarters occurring in 2 days. The disease was readily reproduced by the introduction of the organisms into the non-diseased udder or by swabbing the teat orifice with infected secretions. Whereas in mouse heart-blood the organism appeared in diplococcal form, in the udders of the two cows, one naturally infected and one experimentally infected, it appeared mainly in chains of six to twenty.

II. CONTAGIOUS ABORTION

A. *Diagnosis*

Though diagnosis is still mainly confined to the blood agglutination test much work has been carried out on the ring test using milk and stained antigen. During experiments in two herds Lembke, Koernlein & Frahm(93) found blood titres of 1:25 to 1:64,000 and milk titres of 1:2 to 1:512. Statistical analysis showed a relationship between the agglutination titres of blood and those of the milk. Roepke, Paterson, Driver, Clausen, Olsen & Wentworth (94) used the ring test on milk or cream obtained from dairy plants or creameries and derived from 30,811 herds. The ring test was positive on 264 out of 385 herds (68%) which were considered as being positive by blood tests. Investigation of 107 of the herds which gave a negative ring test showed that

reactor animals were not in production. Blood tests on 1300 herds, which were positive to the ring test when carried out on either milk or cream, showed 54·5% to have one or more infected animals present. Of the remainder 34·5% gave a negative blood test, and 11·0% gave results which were doubtful. Discrepancies tended to be charged against the ring test, and in many instances this was not justified.

Drimmelen(95) describes a three-tube ring test in which non-reacting milk is used to dilute the milk sample and with which he claims that it is possible to distinguish between vaccinated and infected animals. A titre of more than 1:2 is seldom encountered 3–4 months after vaccination, whereas infected cows show high titres for months or even years. Holm, Eveleth & Rheault(96) describe a dilution method for the milk ring test using non-reacting milk to obtain nine dilutions ranging from 1:1 to 1:800. The non-reacting milk used for the dilutions may be preserved with formalin and stored under refrigeration without affecting the test. The technique employs a constant amount of antigen, a decreasing amount of the test milk and an increasing amount of the diluent. The tubes are centrifuged for 10 min. at 1500 r.p.m. before the results are read. Milk from mastitic quarters, colostrum, the secretion from dry cows, and the agglutination inhibition factor present in some types of milk did not appear to interfere with the milk ring test when serial dilutions were used. In order to economize in the use of glassware for the milk-ring test Huddleson & Carrillo(97) have devised a tube rack made in solid plastic with a series of holes bored to act as tubes. They also advocate the use of drinking straws cut into two or more pieces as pipettes. They give full details for the preparation of a strained antigen which will give a reaction with milk as low as 0·5% butterfat. A composite sample from a herd containing one reactor, i.e. a dilution of 1:40 to 1:160, should show a coloured ring of 1·0–3·0 mm. after incubation for 1 hr. The authors have not seen reactions with milk from mastitic cows, but colostrum gave a reaction. King(98) describes a capillary tube method for conducting the *Brucella*-stained antigen milk test, by which milk high in agglutinin content will give a reaction in 5 min. After examining the blood and milk from 428 animals the capillary test seemed to be somewhat superior to the ring test in overall agreement with the blood test. This test consists of allowing 5·0–7·0 mm. of strained antigen to run into a 0·5 × 90·0 mm. tube, and then allowing the tube to fill with test milk. The tube is inverted to mix the contents and is then placed at an angle of 45° from the horizontal. A positive sample shows rings of clumping. Bremer(99) considers that the *Brucella* ring test should be regarded as a screening test to locate infected herds, which could then be subjected to the official blood test.

In fluid taken from the uterine mucosa of cows Jepson & Vindekilde(100) have demonstrated positive agglutination titres varying from 1:5 to 1:1280. In a number of cases the blood titre was less than that of the uterine fluid and was sometimes negative. They collected the fluid by means of a tampon left in the vagina for 1 hr. The fluid expressed from the tampon, usually 3–4 ml., was taken as being a dilution of 1:2·5.

In order to study the incubation period of *Br. abortus*, Thomsen(101) infected ten heifers at the time of service by injecting 10 ml. of a suspension of infected placenta into the prepuce of the bull (a massive dose compared with what would be obtained by natural infection during copulation). Five of the heifers aborted; their placentae showed specific changes, and the presence of *Br. abortus* was demonstrated microscopically. The incubation period was very long, i.e. an average of 225 days. Four of the heifers which did not abort failed to show antibody formation, suggesting resistance to infection at the time of service. Nineteen heifers were infected orally and conjunctivally at different stages of pregnancy, and sixteen of them aborted, the placentae showing typical changes. The incubation period was shown to be longer in animals infected early in pregnancy than in those infected later. It varied from 225 days in early pregnancy to 44 days in late pregnancy. Because of the long periods between infection and the occurrence of demonstrable blood reactions, Thomsen considers that only slight significance can be attached to a single negative blood test in an animal whose origin is unknown. He found that the complement-fixation test agreed well with the agglutination test, but a positive result with the former usually occurred later than with the latter.

Wolff & Dinger(102) describe a strain (L) of *Br. abortus* which possesses, besides A and M,

a further antigen L. This envelops the organism so as to render it inagglutinable by the usual rabbit anti-*Brucella* sera, though it is agglutinable by the sera of *Br. abortus* carriers. Heating to 100° C. for 30 min. renders the organisms agglutinable by rabbit anti-*Brucella* sera. The authors suggest that the L strain might be of value in the detection of carriers. Cox & Kutner (103) state that the rapid macroscopic agglutination test is more reliable than the tube method because agglutinin blocking activity does not seriously interfere with it. They tested the sera of eighty-eight cows from five herds for this activity in addition to carrying out the rapid and tube tests. In two herds, which were considered free from brucellosis, there was no evidence of agglutinin-blocking activity, and with the exception of one vaccinated animal none showed an agglutination titre of over 1:40 by either method. Of the fifty-two animals in three herds considered clinically suspicious, twenty-four showed a tube agglutination titre of more than 1:40, thirty-five showed a macroscopic agglutination titre of more than 1:40, and twenty-five agglutinin-blocking activity. Čulen (104), however, considers that the rapid test is not to be recommended as it gives faulty results in many cases. Comparing the slow agglutination test and the complement-fixation test he obtained results which suggest that the former was slightly superior. He considers that in affected herds an agglutination titre of 1:50 may be regarded as positive. Czarnowski (105) advocates the use of the complement-fixation test to confirm diagnosis when agglutination is positive in a dilution of 1:10. Rice & Avery (106) found that, using constant complement, the conglutinating complement-absorption test was better than the haemolytic complement-fixation test in the diagnosis of brucellosis.

With regard to cutaneous allergy tests for the diagnosis of brucellosis Ottosen & Plum (107) describe the preparation of a purified extract of *Br. abortus*, which when injected intravenously in 1 ml. doses into rabbits did not give rise to agglutinin formation or to the formation of complement-fixing antibodies. Subcutaneous injection of 0·05 ml. doses elicited conspicuous allergic reactions in *Brucella*-infected guinea-pigs, but practically no reaction in normal guinea-pigs, or in guinea-pigs previously exposed to the extract. These findings were largely confirmed in field experiments. Cattle free from brucellosis showed an increase in skin thickness of only 1·0–1·5 mm., whereas infected cattle showed an increase of 6·0–7·0 mm. Mosimann (108) has studied the toxicity and antigenic and sensitizing potency of several fractions prepared from *Br. abortus*. Tested on mice intraperitoneally and on rabbits intracutaneously, he found that the glucolipid polypeptide complex was toxic, and this was true to a slight degree of the aqueous whole extract and the nucleoprotein and polysaccharide fractions. Complete antigenic potency was shown by the aqueous extract and the glucolipid polypeptide complex. He considers that as the polysaccharide fraction, which proved to be a hapten, is suitable for the cutaneous allergy test, it may be of use in the diagnosis of contagious abortion.

B. *Bacteriology*

Levine & Wilson (109) have confirmed that Strain 19 may be differentiated from other strains by its greater sensitivity to the bacteriostatic action of thionine-blue. A concentration of 0·4 mg. of thionine-blue per litre of Bacto-tryptose agar inhibited the growth of thirty-one cultures of Strain 19, whereas sixty-six other strains of high and low virulence grew at this and higher levels of dye. Braun (110) has found that a previously demonstrated heat-stable filterable factor that selectively suppressed the establishment of non-smooth *Br. abortus* variants in buffered broth cultures of smooth types to which normal serum or plasma had been added, was associated with the γ- and certain β-globulin fractions of normal bovine plasma. The factor was also observed in a γ-globulin fraction of human plasma, but, in contrast to the bovine fractions, the human γ-globulins used in these studies exhibited a strong antibacterial effect upon virulent and avirulent strains of *Br. abortus*. Further, Braun (111) has shown that this factor is present in the sera of normal animals of susceptible species (man, cow, rabbit, guinea-pig, pig and cat) and absent from the sera of normal animals of non-susceptible species (hamster, rat, mouse, chicken, quail, duck and pheasant). Serum from animals vaccinated with Strain 19 within 28 months did not suppress non-smooth types, whether the serum contained detectable agglutinins or not. Serum, from animals naturally infected but not vaccinated, when added to S. 6232 (virulent) failed to suppress

non-smooth variants, but it did suppress them when it was added to S. 2583 (from strain 19). Serum from animals which had been vaccinated and naturally infected failed to suppress variants in either strain. These results suggest that the *in vitro* variation of an animal's serum may be utilized for diagnostic tests to indicate the status of an animal in regard to *Brucella* infection or past vaccination, independent of agglutination titres. Cole (112) describes a procedure for the purification of the bovine γ-globulin factor which suppresses non-smooth variants of *Br. abortus*.

Schuhardt, Rode, Foster & Oglesby (113) found an anti-*Brucella* factor in certain preparations of tryptose broth, which proved uniformly toxic to relatively large inocula of each of thirteen cultures of *Br. abortus*. Oxidized polypeptides or amino-acids were suspected as the toxic agents. Schuhardt, Rode & Oglesby (114) tested the nineteen amino-acids of a casein digest at concentrations equivalent to 0·5, 1·0 and 1·5% casein in the basal medium of McCullough & Dick (115) without the ammonium salt. Their results showed that at a concentration of 0·5% the amino-acids served as an acceptable source of nitrogen with little evidence of toxicity for *Br. abortus* 1257. At higher concentrations the medium acquired a toxicity similar to that observed in toxic peptones in that only the largest inocula were able to attain visible growth. Tryptophan and cystine were found to be highly toxic in a medium containing no other amino-acids. Methionine and phenylalanine were slightly toxic, but the other fifteen amino-acids of the casein digest were not found to be toxic at concentrations well above those found in 1–2% casein digests. Only the cystine toxicity persisted in the presence of other amino-acids. Though cystine toxicity for brucellae correlates in many respects the previously reported tryptose toxicity for these organisms, it has not yet been established whether the two are identical. Gerhardt, Levine & Wilson (116) studying the oxidative dissimilation of amino-acids and related compounds found that only three were oxidized or deaminated at appreciable rates by Strain 19 of *Br. abortus*.

Sanders & Huddleson (117) have studied the influence of atmospheric gases on the multiplication of *Brucella*. Using *Br. abortus*, *Br. suis* and *Br. melitensis*, they found that rates of multiplication are greatly increased in liquid culture media when the organisms are grown in an atmosphere of pure oxygen under constant agitation. In the original medium used the constituents were not sufficient to support growth with oxygen and the following medium was substituted: tryptose 3·0 g., glucose 2·0 g., thiamine hydrochloride 0·5 g., sodium chloride 0·5 g., distilled water 100·0 ml. *Br. abortus* did not grow so profusely as the other two species; it is possible that it may need some additional nutritional factor or a different atmosphere or both. It exhibited a much greater tolerance for high concentrations of carbon dioxide than the other two species, which was not unexpected in view of the need of this gas for the primary isolation of *Br. abortus* from pathological material. The same authors (118), using the same medium for growth, found that more glucose was decomposed per unit cell by each of the three species when oxygen was supplied than on growth in stagnant air. In an atmosphere of pure oxygen, thiamine was not required for the initial utilization of glucose but was needed to produce large populations. Thiamine increased both the amount of glucose decomposed and the rate of multiplication with *Br. abortus* and *Br. melitensis*, but *Br. suis* did not require it.

Rottgardt & Pirazzi (119) point out that, as *Brucella* survives at a temperature of 3–5° C. for 80 days or at 37° C. for 8 days even when 1:1000 or 1:2000 crystal violet is added to the culture, cattle used for the production of foot and mouth disease crystal-violet vaccines should be free from *Brucella* infection.

C. *Vaccination*

A valuable contribution on this subject has been made by McDiarmid (120), who compared the use of the intradermal, intracaudal and subcutaneous routes in the production of immunity in cattle by inoculation of strain 19. He inoculated 5·0 ml. subcutaneously, 0·2 ml. intradermally as in the tuberculin test and 1·0 ml. into the fibrous tissue about $\frac{1}{2}$ in. from the tip of the tail, in three groups each consisting of ten animals. A fourth group of twelve animals remained unvaccinated. After they had been in calf for about 5 months the animals were challenged by conjunctival exposure of 150 million virulent *Br. abortus* organisms. As a result ten calves in the

unvaccinated control group were born dead. It appeared that the intradermal and intracaudal inoculations conferred an immunity comparable to that produced by the subcutaneous inoculation of the larger dose of the same antigen. As the author points out, if supplies of vaccine became restricted, the fact that smaller amounts are required by the intradermal and intracaudal routes would give them a great advantage.

Campbell & Rodwell (121) vaccinated twenty-seven heifers as calves with 5 ml. Strain 19 vaccine and exposed them, along with twenty-nine unvaccinated control heifers, to *Br. abortus* infection during pregnancy. Only one of the vaccinated heifers showed evidence of infection by agglutination reaction, but eighteen of the controls became infected, ten of them aborting, thus demonstrating the high degree of protection which can be obtained against infection and abortion by using Strain 19 vaccination. Five of the controls showed evidence of pre-pregnancy natural immunization against *Br. abortus*. Studies on the repeated vaccination of cattle with Strain 19 have been carried out by Berman & Beach (122). Two groups of twenty-two and twenty-one calves were vaccinated when they were about 8 months old and then again when they were either 14 or 20 months old. Following the second vaccination there was a rapid rise in agglutination titre, which fell on an average to 1:100 within 3 months and was 1:25 at the termination of pregnancy. A control group of cattle which was vaccinated only once showed a typical return to negative status within 12 months. Jakubowski (123) reports confusing results after the vaccination with Strain 19 prepared in Poland of 245 cattle which gave negative tests seriologically. He states that the agglutinin reaction became positive, but the titres were confusing and did not agree with the reports of other authors. He considers that Polish cattle do not react in the same way as American cattle. Maderious & Albaugh (124) report a marked reduction of brucellosis in cattle in Monterey County, California, as a result of an experimental vaccination programme covering 7 years. Holzer (125) stresses the importance of hygiene and the isolation of infected animals in the control of brucellosis. Where it is impossible to eliminate all reactors immediately, vaccination of non-infected heifers 6–8 months old with Strain 19 is indicated. He considers the vaccination of positive reactors useless. Thomson (126) has studied the blood tests for brucellosis under two control plans. In plan A the herds were blood tested and the calves were vaccinated with Strain 19, and in plan B the calves were vaccinated, but the herds were not blood tested. Herds for plan A were recruited from the herds used in plan B. Reactors showed evidence that under plan A there was a high degree of protection in cows which were between 2 and 5 years of age. Of calves vaccinated under plans A and B, 8·5% reacted at 2–3 years old, 5·0% due to field infection and 3·5% due to vaccination. Moore & Mitchell (127) vaccinated sexually mature cows with Strain 19 vaccine. Of 188 non-pregnant cows which were vaccinated fifty-three became infected, and of 181 vaccinated pregnant cows thirty-seven became infected.

Jordan (128) reports the use of strain 45/20 McEwan and Strain 19 during a 7-year period in a self-contained herd. Significant titres did not develop from the repeated use of strain 45/20, but they did with Strain 19. In vaccinated adults positive titres persisted up to 4 years, but in calves only up to the first calving. The incidence of abortion in the herd was low and attributable to causes other than natural brucellosis or vaccination. Fertility was high, the percentage of animals calving varying from 88 to 100. Manthei & Carter (129) found that in cows artificially infected with virulent *Br. abortus* by way of the conjunctiva, bacteraemia was more persistent in highly susceptible unvaccinated cattle than in those vaccinated with Strain 19. Cows vaccinated with 45/20 vaccine showed a high incidence of bacteraemia, a high abortion rate and a high incidence of infection. At the end of gestation, however, *Br. abortus* was found in thirty-three unvaccinated controls, in five vaccinated with Strain 19, but in none vaccinated with 45/20 vaccine. Huddleson (130) has found that certain mucoid phases of *Br. suis* prepared as a vaccine (*Brucella* M vaccine) when injected into guinea-pigs produce a high degree of resistance to the three species of *Brucella*, but large numbers of such live cells were incapable of producing progressive infection either in guinea-pigs or in heifers. Injection into adult cattle does not produce an agglutination titre for the smooth-phase antigen higher than 1:100, and this titre is not retained after 90 days. Thus the development of an agglutination titre within 3–4 months

after vaccination would indicate natural infection. In twenty-two herds with evidence of brucellosis, of 722 negative adults injected with M vaccine and left exposed to more than 100 infected cattle, only twenty-three became infected in a period of 12–14 months.

After the introduction of cattle vaccinated with Strain 19 into a herd in Singapore, which was originally made up of double agglutination tested animals, Aun, Wright & Sen(131) found 40% of the herd had agglutination titres up to 1:25. Further tests with Weybridge standard *abortus* antigen (concentrate) showed that a high proportion were infected. Organisms were isolated from two cases on liver infusion agar with gentian violet 1:250,000.

In addition to work on live vaccines the effects of adjuvants on vaccines prepared from dead organisms have also been studied. Using a vaccine produced by absorption of killed *Br. abortus* on aluminium hydroxide, Hausmann, Flatken & Ruge(132) found in cattle that one inoculation of 20 ml. containing 10 billion bacteria/ml. was followed in 8 days by an agglutination titre of 1:800, and that it increased during the following 2 weeks to 1:1600. Live(133) also found that adjuvants prolonged the state of resistance in guinea-pigs when used with ether-killed *Br. abortus*. He found that a saline-in-oil emulsion was more efficacious than potassium alum. Live & Danks(134) investigated the antigenicity and immunizing properties of a saline-in-oil emulsion of ether-killed *Br. abortus* in cattle and compared them with the corresponding properties of Strain 19 vaccine. In a herd in which brucellosis had existed for about a year seventy-three cattle, negative to the agglutination test and in different stages of the reproductive cycle, were divided into three groups. In the first group, inoculated with Strain 19, the mean agglutination titre reached its peak in 2 weeks and then declined rapidly. Eight animals in this group aborted. In the second group, inoculated with ether-killed *Br. abortus* in a saline-in-oil emulsion, the titres increased gradually and remained at their height for some time and then decreased. It appeared that the emulsification in oil retarded the absorption of the antigen. A second inoculation 6 months after the first was followed by a sharp rise in agglutinin production. One animal in this group aborted. In the third group of unvaccinated controls one animal aborted. A challenge experiment was carried out, but on too small a scale for definite conclusions to be drawn. Two heifers from each group were challenged with 166×10^4 *Br. abortus* organisms, and one of the two controls became infected. Larson(135) was able to produce immunity in 40–70% of the guinea-pigs he used by inoculating them with a soluble antigen obtained from *Br. abortus* by ether extraction. The animals were challenged with about 1000 live organisms of the strain. The addition of Freund's adjuvant failed to increase the immunizing power, but the addition to the antigen of heat-killed human tubercle bacilli incorporated in Freund's adjuvant, increased the power so that 80–100% of the experimental animals became immune.

D. *General*

Donker-Voet(136) estimates the incidence of *Br. abortus* infection in Indonesia as 5% in the years 1948–9, a similar proportion to the years 1938–40. Zintek(137) reports that since 1934, the testing of more than 7 million cattle in Wisconsin indicated that 20–30% of all herds and 5–6% of all cattle are infected. Rossi & Cedro(138) studied the incidence of brucellosis in the municipal slaughterhouse and packing house of the city of Buenos Aires by the agglutination test. Of 4966 cattle tested 18·1% were positive, of 5377 hogs 20·3% were positive and of 5510 sheep 7·3% were positive. Reactions in the sheep were of doubtful significance since forty-three attempts to isolate the organism failed. Of the total personnel 40% were positive to the serum agglutination test and 15% had the disease in its clinical form, but most of the affected people worked on the hog-killing floor. During the years 1938–48, Hess & Brunner(139) examined 9261 cases of bovine abortion. A diagnosis of infection was established in only 4106 of these cases. Infection in 2973 of the cases was brucellosis, in 840 trichomoniasis, in 245 *Corynebacterium pyogenes* and in forty-eight streptococcal. A few staphylococcal and miscellaneous fungus infections were also found. Dysfunction of the sexual organs was considered responsible for some of the 5155 non-infectious abortions. The tendency for either infectious or non-infectious abortion to occur was greatest between the eighth and thirteenth weeks of pregnancy and from the sixth month to full term.

Kaay(140) reports the infection with *Br. abortus* of the sex organs of two bulls which were being used for artificial insemination, and Manthei, Detray & Goode(141) recovered this organism from the semen of a bull for eighty successive ejaculations. The latter authors artificially inseminated twelve susceptible cattle with the infected semen, and eight of them (six virgin heifers and two cows) developed active brucellosis. Christiansen(142) reports that six heifers, kept for 5 days or more on pasture where animals excreting large numbers of *Br. abortus* had been grazing, failed to develop an agglutination reaction. This was true also of heifers which grazed for several months in a yard separated from aborting animals by a single or double electric fence, but of four heifers drinking the same water as the aborting cattle, one became infected. Ten heifers were watered once from a pond polluted with the after-birth and vaginal discharge from aborting animals, and two of them developed an agglutination reaction. *Brucella* organisms were recovered from the water 8 days after pollution but not after 30 days.

Domanski(143) states that observations on various ranches in Poland suggest that the most important factor in the aetiology of brucellosis in cattle is the virulance of the organism and that neither diet nor sanitary measures appear to be so important. Balducci & Mangione(144) report that *Brucella* infection shows a less severe course in vitamin C-deficient guinea-pigs and in vitamin B-deficient rats than in normal animals, and that it does not accelerate the onset of scurvy or beri-beri.

Of the numerous papers which have been published in recent years on the subject of brucellosis in the human subject, two are directly concerned with bovine brucellosis. Braude, Gold & Anderson(145) report that, after feeding heat-killed *Br. abortus* strain 1257 suspended in milk or water to sixty-seven human volunteers, agglutinins appeared in the sera of 23·9% with titres up to 1:640. No dermal hypersensitivity was produced. Fleming & Roepke(146) state that there was no appreciable increase in the number of human cases of brucellosis reported during the period 1937–47 in a district of twenty-one counties in Minnesota, which was under a control plan. In the same period the number of cases reported in districts not under the control plan increased by four to ten times.

E. *Treatment*

Studies of the effect of streptomycin and sulphadiazine in experimental brucellosis in guinea-pigs have been made by Holm & McNutt(147). Male guinea-pigs were inoculated with about one million *Br. abortus* strain 2308 and were treated with sulphadiazine (120 mg. by mouth), with streptomycin (5 mg. subcutaneously), with sodium *p*-aminobenzoate or with various combinations of the three. With one exception treatment with a combination of sulphadiazine and streptomycin produced bacteriological cures in 80–100% of the animals experimentally infected. The combination was as effective 10 days after inoculation as when given on the day of infection, and as effective when given once as when given five times a day. Braude & Spink(148) found that a combination of streptomycin and sulphadiazine afforded excellent protection in mice experimentally infected with *Brucella*, and that similar protection was given by aureomycin. Holm & Moore(149) showed that a combination of aureomycin and sulphadiazine given subcutaneously and orally proved to be effective in reducing spleen size in experimental brucellosis in guinea-pigs, but the combination was not as effective as streptomycin and sulphadiazine in eliminating infection. The former combination produced a toxic effect which was more marked than that produced by aureomycin alone. Using the calcium chloride complex of streptomycin in 0·5 ml. doses subcutaneously and the sodium salt of sulphadiazine in 0·5 ml. oral doses, Holm & Moore(150) have shown, in experimental brucellosis in guinea-pigs, that treatment every other day produced as high a percentage of sterilizations as daily treatment. The authors are of the opinion that treatment every other day would not be as likely to give rise to resistant strains as daily treatment.

From the above results it appears that brucellosis therapy is successful in laboratory animals, but the limited amount of work reported on the treatment of brucellosis in cattle is not encouraging. Larsen & Gilman(151) treated four cows, acutely affected with brucellosis, with initial doses of 5·0 g. aureomycin/1000 lb. body weight followed by doses of 5·0 or 2·5 g. twice

daily which were continued in three of the animals for 6 weeks. Guinea-pig inoculation and serum and whey agglutination tests showed that the animals were still infected at least 2 months after treatment. They concluded that aureomycin failed to arrest the disease or to modify its course as observed in untreated animals. Watts, Boley & Greig(152) repeatedly treated four cattle, which were naturally infected with brucellosis, with sulphamethazine and transfusions of normal citrated bovine whole blood or normal serum. Three infected cattle were held as controls. Haemologic studies were made before, during and after treatment. The treatment had no apparent effect on the blood titres and did not prevent three cows from shedding *Brucella* in their milk. Seelemann & Meyer(153) however, report good results with streptomycin against brucellosis in cattle.

P. S. Blackburn

The Hannah Dairy Research Institute
Kirkhill, Ayr, Scotland

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